THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR ..... Alice Hontela .....

TITLE OF THESIS Daily cycles of serum gonadotropin in the goldfish, *Carassius auratus*: effects of temperature, photoperiod, sexual condition, pinealectomy and blinding

DEGREE FOR WHICH THESIS WAS PRESENTED ..... Master of Science .....

YEAR THIS DEGREE GRANTED ..... 1978 .....

THE UNIVERSITY OF ALBERTA

# DAILY CYCLES OF SERUM GONADOTROPIN IN THE GOLDFISH, *CARASSIUS AURATUS*: EFFECTS OF TEMPERATURE, PHOTOPERIOD, SEXUAL CONDITION, PINEALECTOMY AND BLINDING

by

© ALICE HONTELA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SPRING, 1978

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Daily cycles of serum gonadotropin in the goldfish, *Carassius auratus*: effects of temperature, photoperiod, sexual condition, pinealectomy and blinding," submitted by Alice Hontela in partial fulfilment of the requirements for the degree of Master of Science.

## ABSTRACT

The effects of photoperiod, temperature, sexual condition, pinealectomy and blinding on daily cycles of serum gonadotropin (GTH) in the goldfish, *Carassius auratus*, were investigated.

In the daily rhythm experiment, fish were subjected to four different experimental regimes of photoperiod and temperature (16L:8D/21±1°C; 8L:16D/21±1°C; 16L:8D/12±1°C; 8L:16D/12±1°C) at different times of the year. Fish with a regressed gonad (GSI = 1.1±0.1%) were used in September 1976, female fish undergoing ovarian recrudescence (GSI = 5.2±0.4%) were used in January 1977, and female fish with a mature ovary (GSI = 11±0.7%) were used in March 1976. In the pinealectomy-blinding experiment, five experimental groups (pinealectomised, blinded, blinded and pinealectomised, sham operated and intact) were established in October 1976 with regressed fish (GSI = 2.7±0.2%) and in April 1977 with mature female fish (GSI = 8.5±1.2%). Fish in this experiment were subjected to 16L:8D/21±1°C.

Blood samples were taken throughout the 24 hour period in the daily rhythm experiment, and at 1200 hr and 2000 hr in the pinealectomy blinding experiment. Serum GTH levels were measured by radioimmunoassay.

Regressed fish did not show daily variations in serum GTH levels under different photoperiod and temperature regimes or showed variations smaller in magnitude than those of females undergoing ovarian recrudescence or mature females.

The pattern of daily rhythm of GTH levels under the same set of environmental conditions was somewhat similar in the latter two groups

and although the GTH levels of the females undergoing ovarian recrudescence and mature females were higher than the GTH levels in regressed fish most of the time, during a portion of the 24 hour period the GTH levels of the three groups were similar. Pinealectomy and blinding did not affect the daily variations in GTH levels in fish with a regressed gonad. Pinealectomy seemed to abolish daily variations in serum GTH levels in mature females, but further investigations are necessary before this can be established with certainty.

The results indicate that serum GTH levels in regressed fish are relatively uniform throughout the 24 hour period and are not greatly influenced by either different conditions of photoperiod and temperature, or pinealectomy and blinding. The GTH levels detected in these fish seem to represent a basal level of GTH secretion.

Serum GTH levels in female fish undergoing ovarian recrudescence and mature female fish fluctuate during the 24 hour period and the pattern of these fluctuations varies depending on the photoperiod and temperature regime. Both temperature and photoperiod seem to be important factors in regulation of daily cycles of serum GTH. The variations in serum GTH levels in these two groups seem to represent fluctuations above the basal level of serum GTH. Pinealectomy seems to abolish these fluctuations in mature female fish.

## ACKNOWLEDGEMENTS

I would like to thank Dr. R.E. Peter for the supervision of this project. I am very grateful for his suggestions, financial support, his help with the blood sampling and the radioimmunoassay, and his assistance in the preparation of this manuscript. I would particularly like to thank him for his constant interest and encouragement.

I would like to thank my advisory committee members, Drs. J.K. Lauber and W.C. Mackay for their guidance. I also thank Drs. D.D. Beatty, P. Crockford, J.K. Lauber and A.N. Spencer for their critical review of the manuscript.

I am grateful to Christine Bohaichuk, Ron Koss, Ann Kyle and Murray Wiegand for their help with the blood sampling.

I would like to thank Joe Rasmussen for his interest in this project and his help with the statistical analysis.

I would like to acknowledge the financial support provided by the University of Alberta in the form of graduate teaching assistantships and intersession bursaries.

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# INTRODUCTION

One of the best known aspects of the reproductive cycles of temperate zone teleost fishes is the fact that they are seasonal breeders and that they adjust their physiology in response to the changing environment (for review see de Vlaming, 1972). However, the basic question of which environmental cues a fish may use and the mechanism for this still remain largely to be answered.

The trophic influence of the pituitary gland over the gonadal development in fishes is now well established and the effects of hypophysectomy on the gonads have been reviewed by several authors (Pickford and Atz, 1957; Dodd, 1960; Ahsan and Hoar, 1963; Hoar, 1965). In general, the gonads of hypophysectomised fish show regressive changes which can be reversed by administration of gonadotropin hormones (GTH) or pituitary extracts (Pickford and Atz, 1957). Purified carp (*Cyprinus carpio*) GTH can induce vitellogenesis (Burzawa-Gerard, 1974) or reinitiate spermatogenesis in the hypophysectomised goldfish *Carassius auratus* (Billard *et al.*, 1970). It also maintains yolky oocytes and causes ovulation in gravid hypophysectomised Indian catfish *Heteropneustes fossilis* (Sundararaj *et al.*, 1976 . Ahsan (1966) reinitiated spermatogenesis in hypophysectomised lake chub *Couesius plumbeus* by treating the fish with pituitary extracts of salmon (*Oncorhynchus tschawytcha*, *O. kisutch*) or mammalian luteinizing hormone (LH). The stimulatory action of GTH has been demonstrated also by experiments using methallibure which is known to inhibit GTH in mammals. Pandey (1970) showed that methallibure blocked ovarian or testicular differentiation in juvenile guppies *Poecilia reticulata*

and inhibited spermatogenesis in adult fish (Pandey and Leatherland, 1970). Methallibure also inhibited spermatogenesis and testicular steroidogenesis in the seaperch *Cymatogaster aggregata* (Wiebe, 1968) and suppressed plasma and pituitary GTH levels and spermatogenesis in the goldfish (Breton *et al.*, 1973). Data provided by Breton and co-workers indicated that methallibure could block the release of GTH from the pituitary, and perhaps also GTH synthesis in the pituitary, since there was no apparent accumulation of GTH in the pituitary following the blockage of release. This provides some indirect evidence for the existence of a hypothalamic gonadotropin releasing factor (GRF) in the goldfish.

More direct proof for the existence of GRF has been provided by Crim *et al.* (1976) who demonstrated that GTH secretion could be stimulated by intraventricular injection of hypothalamic extracts in goldfish. Similar work has been done in another cyprinid, the carp (Breton and Weil, 1973; Weil *et al.*, 1975; Breton *et al.*, 1975). Synthetic LH-FSH/RH (luteinizing hormone-follicle stimulating hormone/releasing hormone) also stimulates plasma GTH levels in carp (Breton and Weil, 1973; Weil *et al.*, 1975) and induces ovulation in the goldfish (Lam *et al.*, 1975). Evidence for the existence of GRF in fish has also been provided by lesioning experiments. Peter (1970) showed that lesions in the nucleus lateralis tuberis (NLT) pars posterior and the posterior part of NLT pars anterior of the hypothalamus caused a decrease in the gonadosomatic index

$$\left(\text{GSI} = \frac{\text{weight of gonads}}{\text{body weight}} \times 100\%\right)$$

in the goldfish. It appears that these specific areas of the fish

hypothalamus are involved in the secretion of GRF. These results are also supported by studies which correlated cytological signs of activity in the NLT and stages of gonadal development in teleost fishes (Polenov, 1950; Brehm, 1958; Billenstein, 1962; Dixit, 1967; Honma and Suzuki, 1968; Zambrano, 1971; Viswanathan *et al.*, 1974; Anand *et al.*, 1974).

Little information is available to clarify the mechanisms by which the brain controls the secretion of GRF. The negative feedback effect of the sex steroids at the hypothalamo-pituitary level seems to be one of the factors influencing GTH secretion. Administration of steroids inhibited the development of gonads in the goldfish, *Carassius auratus* (Billard, 1974), the Indian catfish, *Heteropneustes fossilis* (Sundararaj and Goswami, 1968) and medaka, *Oryzias latipes* (Egami, 1954). On the other hand, plasma GTH levels increase after castration in rainbow trout *Salmo gairdneri* (Billard *et al.*, 1976) and Goswami and Sundararaj (1968) demonstrated that following a unilateral ovariectomy in the Indian catfish, there is compensatory growth of the remaining ovary. This suggests that a greater GTH secretion occurs following a decrease in the sex steroids level. Clomiphene citrate, a structural analog of estrogens which acts in competition with estrogens, induced a GTH discharge in carp (Breton *et al.*, 1975a), and accelerated ovulation in the loach, *Misgurnus anquillicaudatus* (Ueda *et al.*, 1976) and the goldfish (Pandey *et al.*, 1973), possibly by causing increased GTH secretion.

The sites where the negative feedback exerts its effects seem to be the pituitary and the NLT region in the hypothalamus. Billard and Peter (1977) showed that serum GTH levels in the goldfish increase

following implantation of anti-estrogens in the NLT region or in the pituitary. Pfaff *et al.* (1975) demonstrated that after injection of radioactive testosterone into green sunfish *Lepomis cyanellus*, the radioactive hormone could be detected in the NLT region and in the pars distalis of the pituitary. Investigations correlating cytological signs of increased activity of pituitary gonadotrophs following castrations (McBride and Von Overbeeke, 1969; Febre and Lafaurie, 1971) or decreased activity of gonadotrophs following administration of sex steroids (Van Overbeeke and McBride, 1971; Febre and Lafaurie, 1971) suggest that the pituitary is a control site where the negative feedback might act. Similar studies concerning the hypothalamus have also been done (Dixit, 1970; Zambrano, 1971; de Vlaming, 1974). This evidence and also the data reported by Peter (1970) suggest that the NLT region of the hypothalamus and the pituitary are the sites involved with the control of GTH secretion.

The fact that temperate zone teleosts are seasonal breeders indicates that various environmental factors may have input to the hypothalamus to influence GRF secretion. Both temperature and photoperiod appear to be important factors involved in the regulation of reproductive cycles in fish. It should be noted that the effect of these two parameters varies depending on the species studied (for review see de Vlaming, 1972, 1974; Peter and Hontela, 1977). In medaka *Oryzias latipes*, reduced photoperiod lowered the GSI (Urasaki, 1972). Henderson (1963) demonstrated that photoperiod is the dominant factor in regulation of reproductive cycles of the brook trout *Salvelinus fontinalis*. In the estuarine gobiid fish *Gillichtyes mirabilis*, gonadal recrudescence

occurred at temperatures from 10°-20°C and on short or long photoperiods (de Vlaming, 1972a). Viswanathan *et al.* (1974) and Anand *et al.* (1974) demonstrated that long photoperiod and warm temperature (25°C) induced ovarian recrudescence in the Indian catfish *Heteropneustes fossilis*. Vasal *et al.* (1976) further demonstrated that warm temperature was the main factor in the regulation of ovarian recrudescence in the catfish, regardless of photoperiod. De Vlaming (1975) investigated the effect of photoperiod and temperature in the golden shiner *Notemigonus crysoleucas*, a member of the cyprinid family. It seems that temperature plays the dominant regulatory role in the reproduction of *Notemigonus*, although since the effects of temperature and photoperiod are dependent on season in this species, it is difficult to make any generalisations. A dominant role of temperature in regulation of gametogenesis has been shown in another cyprinid, the lake chub *Couesius plumbeus* (Ahsan, 1966a).

The relative importance of photoperiod and temperature has not been demonstrated in cyprinids such as the carp or goldfish. Gillet *et al.* (1977) studied the effects of temperature on the plasma and pituitary GTH levels and spermatogenesis in the goldfish *Carassius auratus*. They showed that in April and May, the plasma GTH levels of fish held at a temperature of 17°C or above, were greater than plasma GTH levels of fish held at 10°C. In April, the pituitary GTH levels were higher at temperatures above 17°C than at temperatures below 17°C, whereas in June the opposite was true. Pituitary GTH levels were higher at temperatures below 17°C than at temperatures above 17°C. They also showed that 17°C and 24°C stimulated spermatogenesis in

comparison with fish held at 10°C, while 30°C inhibited spermatogenesis. The fish in these experiments were exposed to natural or simulated natural photoperiod, so effects of photoperiod cannot be determined. However, it is apparent that temperature has important influences on GTH secretion.

Information concerning the effects of photoperiod and the route of its influences in teleosts is scarce. The pineal organ of certain teleosts is a light receptive organ (Grunewald-Lowenstein, 1956; Dodt, 1963; Kappers, 1965; Morita, 1966; Takahashi, 1969) and it contains secretory cells responsive to light (Pflugfelder, 1956; Hafeez and Ford, 1967; Rüdeberg, 1971). The existence of an indolamine metabolism has also been shown (Quay, 1965; Oguri *et al.*, 1968; Fenwick, 1970; Urasaki, 1974). In mammals, melatonin, an important indolamine of the pineal gland, is mostly synthesised in the absence of light (Axelrod and Wurtman, 1965; Wurtman *et al.*, 1968) and its antigonadotropic effect has been demonstrated in the rat (Frashini *et al.*, 1968; Vaughan *et al.*, 1971; Kamberi *et al.*, 1971). Fenwick (1970) determined that the amount of melatonin stored in the pineals of immature king salmon *Oncorhynchus tschawytcha* was 6 times greater than the amount of melatonin stored in pineals of mature individuals. These results suggest a possible antigonadotropic effect of melatonin. Fenwick (1970a) also studied the effect of pinealectomy in the goldfish at different times of the year. Pinealectomy had no effect most of the year but if performed prior to the spawning season, the GSI of the operated fish increased significantly. Melatonin treatment in the same fish inhibited this increase in GSI (Fenwick, 1970). Sundararaj

*et al.* (1976a) demonstrated an antigonadotropic effect of melatonin in the catfish *Heteropneustes fossilis*. On the other hand, Peter (1968) did not find any effect of pinealectomy on the GSI in the goldfish. The study by Fenwick (1970a) and also investigations carried out by Urasaki (1972a,b) and by de Vlaming (1975a, 1977) suggest that the function of the pineal organ in the control of the reproductive system of teleosts might be different at different times of the year or under different temperature and light regimes.

Although some evidence correlating the environment and the process of gonadal maturation has been presented, relatively few studies correlating gonadal maturation and GTH levels are available. Crim *et al.* (1975) measured the GTH levels in several species of salmonids and presented some evidence for the fact that the process of gonadal maturation can be related to increasing GTH levels. He also proposed that a surge of GTH induces ovulation in these fish. This hypothesis is supported by evidence from studies using also other species of teleosts (Yamazaki, 1965; Breton *et al.*, 1972; Nagahama, 1973); however, the environmental or physiological cues to induce the surge of GTH are unknown. Some indirect evidence suggesting that the environment might be an important factor was provided by Stacey and Pandey (1975), who found that ovulation was induced in gravid female goldfish by increasing the temperature to 20°C.

It is apparent that information concerning the effects of the environment directly on GTH secretion in teleosts is scarce. One approach which might be used to analyse the effect of temperature and photoperiod on GTH secretion in fish would be to measure the GTH levels

over a 24 hour period under different conditions of temperature and photoperiod. The overall effect of the environment could be mediated by changes in GTH secretion in short time periods such as 24 hours.

Little information concerning the diurnal fluctuations of pituitary hormones in teleosts is available. Leatherland *et al.* (1974) demonstrated a circadian rhythm of plasma prolactin and growth hormone in the Kokanee salmon *Oncorhynchus nerka*. Evidence for a circadian rhythm of plasma prolactin in the goldfish has been presented by Leatherland and McKeown (1973) and McKeown and Peter (1976) who also investigated the effects of photoperiod and temperature on this rhythm.

Only two studies are available concerning the daily rhythm of GTH in teleosts. O'Connor (1972) demonstrated a daily rhythm of pituitary GTH content in the prespawning period in the brook trout and the rainbow trout. Since he exposed the fish to only one photoperiod and only one temperature, this study does not provide any insight regarding how temperature and photoperiod may affect the pituitary content of GTH or GTH secretion. Breton *et al.* (1972) found daily fluctuations in plasma GTH in goldfish. However again, only one photoperiod was used and the effects of natural daily variations in temperature were not differentiated from the effects of the light exposure.

Investigations reported hereafter will attempt to determine whether a daily rhythm in serum GTH levels exists in the goldfish *Carassius auratus*, and how the pattern of this rhythm is affected by different light and temperature regimes, and the stage of gonadal maturity of the fish. The effects of pinealectomy and blinding on the daily rhythm in GTH levels, and whether this effect depends on the stage of gonadal maturity of the fish, will also be investigated.

# MATERIALS AND METHODS

## I. Experimental Animals

Goldfish, *Carassius auratus* (common or comet variety, standard body length 2½-3 inches) were purchased from a commercial fish supplier (Grassyfork Fisheries Co., Inc., Martinsville, Indiana). Upon arrival, the fish were sexed by external examination of the pectoral fins (characteristic outgrowths or tubercles are present on fins of males), and by gently squeezing the bellies and noting excretion of either sperm or eggs. It is not possible to externally sex fish with gonads in a quiescent immature state in the late summer or fall, therefore both sexes were used in the fall experiments. The selected fish were held in flow-through aquaria (4800 l) in the main room of the aquatic facilities of the Department of Zoology for a period of 14-21 days before use in the experiments. The temperature was maintained at 13.5 ± 1.5°C and room lights were regulated to give a simulated natural photoperiod. Fish were fed *ad libidum* twice a day with commercial fish food (Ewos, Astra Chemical Ltd., Mississauga, Ontario) treated with terramycin (2:100, Poultry formula, Pfizer Co. Ltd., Montreal, Quebec). After this initial acclimation period, the fish were transferred to experimental tanks and were used in either the daily rhythm experiments or pinealectomy-blinding experiments.

## II. Daily Rhythm Experiments

Three groups of fish were used in this experiment. Females with mature ovaria, having an average gonadosomatic index

$$(GSI = \frac{\text{weight of gonads}}{\text{body weight}} \times 100\%)$$

of about 11% were used in the spring (March 1976). Fish of both sexes with immature gonads in a quiescent state (GSI of about 1%) were used in the fall (September 1976), and females undergoing ovarian recrudescence (GSI of about 5%) were used in winter (January 1977). A diagrammatic outline of the protocol followed in these experiments is presented in Figure 1.

*Photoperiod and temperature regimes*

Following the initial acclimation to the laboratory, fish were transferred into 380 l experimental flow-through aquaria. Four aquaria, each containing 40 fish, were used. Experiments using tanks 1 and 2 (see Fig. 1) were done simultaneously, and within 7-14 days, 80 more fish were taken from the main room tank and the procedure was repeated using tanks 3 and 4 (see Fig. 1). The initial holding regime in each tank was 12 ± 1°C and a photoperiod of 12 hr light:12 hr darkness (12L:12D). The light phase started at 0800 hr and ended at 2000 hr (15 watt, cool white fluorescent light bulb was used). After acclimation to these conditions for 8 days, the experimental conditions were imposed (designated as day 1 on Fig. 1). Fish in tank 1 were subjected to 16L:8D/21±1°C, fish in tank 2 to 8L:16D/21±1°C, fish in tank 3 to 16L:8D/12±1°C, and fish in tank 4 to 8L:16D/12±1°C. The temperature of 21°C was obtained by slowly raising the temperature over a period of 24 hours, starting in the morning of day 1. Lights were automatically turned on at 0800 hr in all tanks, and off at 2400 hr or 1600 hr in the 16L:8D and 8L:16D regimes, respectively. Blood samples were taken

Fig. 1. An outline of the protocol for the daily rhythm experiments.

# Outline for Method - Daily Rhythm

March 1976
Mature
(GSI = 11%

September 1976
Regressed
(GSI = 1%)

January 1977
Maturing
(GSI = 5%)

natural environmental conditions - Indiana

↓

Main Room Tank
12 - 15°C
"Natural" photoperiod
2 – 3 weeks

↓

Experimental Tanks
12°C
12L:12D
8 days

16L:8D
21°C
Tank 1

8L:16D
21°C
Tank 2

Day 1

16L:8D
12°C
Tank 3

8L:16D
12°C
Tank 4

↓

Presample at 20 hr
Day 4

↓

Sample at 8 hr
Day 7 20 hr

↓

Sample at 4 hr
Day 8 16 hr

↓

Sample at 12 hr
Day 9 24 hr

RIA for gonadotropins (serum)
Histology of gonads

during exposure to the experimental temperature and photoperiod regimes, which lasted for 9 days.

*Blood samples*

A presample of blood was taken from the 80 fish at about 2000 hr (start at 1930 hr, end at 2030 hr) on day 4 of the experimental holding regime (see Fig. 1). Starting on day 7, a second sample was taken at 0800 hr and 2000 hr, on day 8 a sample was taken at 0400 hr and 1600 hr and on day 9 a sample was taken at 1200 hr and 2400 hr. (See Section III for detailed description of the sampling procedure). At each sampling time 5-7 fish from one tank were netted at 10 minutes to the hour. Following anaesthetisation, blood was taken from them in a random order. On the hour, 5-7 fish were netted from the other tank and sampled in a similar fashion. The sampling of this second set of fish was usually completed within 10 minutes after the hour. At the end of the procedure, the sampled fish were killed by severing the spinal cord posterior to the head. The fish were weighed and the gonads were dissected out. The body weight, the weight of the gonads and the sex were noted. A small section taken from the middle region of the gonads was then fixed in Bouin's solution. Standard wax embedding was carried out. Sections were stained with hematoxylin and eosin.

## III. Blood Sampling Procedure

Prior to blood sampling, fish were anaesthetised by immersion in a 1:1000 ethyl-m-aminobenzoate solution (Kent Labs., Vancouver, B.C.) in dechlorinated tap water. Fish were considered anaesthetised when

opercular movements were almost undetectable and they lost their ability to stay upright. Fish which were sampled during the dark phase of the photoperiod were anaesthetised in the dark; however, the blood was taken under light. At the time of taking the presample of blood, fish were weighed and identified by a numbered tag (1005 Monel size 1, National Band and Tag Co., Newport, Kentucky), clipped to their operculum. Blood was taken from the caudal vasculature with a 23- or 25-gauge needle fitted to a 1 ml disposable syringe. If the sample being taken at the time was the presample, fish were then transferred into a bucket of dechlorinated tap water and subsequently back into the tank. Fish that did not recover after about three minutes were revived by moving the lower jaw in a simulated respiratory action to force water to circulate over the gills. If the blood being taken at the time was the second sample, the fish was sacrificed after sampling.

Samples were kept over cracked ice and allowed to clot for 1-1½ hr. Serum was separated from the clot by centrifugation at 2,400 RPM for 20 minutes. Using a Pasteur pipette, serum was transferred into 0.4 ml plastic tubes; each tube contained serum from one fish. A volume of 1 µl of 1% thiomerosolate solution (Sigma Chemical Co., St. Louis, Mo.) was added to each tube to prevent bacterial degradation. The samples were then immediately frozen on dry ice and stored at -28°C until assayed for GTH.

## IV. Pinealectomy-Blinding Experiment

Two groups of fish were used in this experiment: fish of both sexes with the gonad in an immature quiescent state (October 1976) and

females with a mature ovary (April 1977). An outline of the protocol for the experiments is presented in Figure 2.

*Photoperiod and temperature regimes*

After 14 days in a flow-through holding tank, the fish were divided into five experimental groups and surgery was performed (see Section V for detailed description of the operations). The five groups were: blinded, pinealectomised, blinded and pinealectomised, sham operated and intact fish. All fish were identified by a numbered tag. The fish were kept in three 380 l flow-through aquaria, each containing 40 fish, 8 fish from each of the five experimental groups. Three tanks were used because of a limited number of available aquaria and the fish from the different experimental groups were distributed as described above, in order to prevent variability between aquaria. The holding regime in all aquaria was 15 ± 1°C and 12L:12D for 7 days. Then the fish were subjected to 12 ± 1°C and 12L:12D regime for 8 days (see Fig. 2). After this acclimation period, the experimental holding regime was imposed (designated as day 1 on Fig. 2). Fish were exposed to 21 ± 1°C and 16L:8D for 9 days. The light phase started at 0800 hr and ended at 2400 hr. Blood samples were taken during the experimental holding regime as indicated below.

*Blood samples*

A presample of blood was taken from all the fish at about 2000 hr (start at 1930 hr, end at about 2030 hr) on day 4 of the experimental holding regime. On days 7, 8 and 9, a second sample was taken at 1200 hr and 2000 hr (see Fig. 2). At each of these individual sampling times, 10 fish were netted at 15 minutes to the hour, anaesthetised and

Fig. 2. An outline of the protocol for the pinealectomy-blinding experiments.

# Outline for Method: Pinealectomy-blinding experiment

April 1977
Mature
GSI = 8.5%

October 1976
Regressed
GSI = 2.7%

natural environmental conditions - Indiana

↓

Main Room Tank
12 - 15°C
"Natural" photoperiod
3 weeks

↓

Environmental Tanks

Surgery

12 L : 12 D / 15°C
7 days

↓

12 L : 12 D / 12°C
8 days

↓

Day 1
16 L : 8 D
21°C

↓

Day 4
Presample at 20 hr

↓

Sample at 12 hr
Day 7 20 hr

↓

Sample at 12hr
Day 8 20hr

↓

Sample at 12hr
Day 9 20hr

RIA for gonadotropins (serum)
Histology of gonads

then a blood sample was taken. On the hour, the same procedure was repeated with 10 more fish. All the fish were caught in a random fashion; however some fish were selectively kept in order to assure the sampling of about 4 fish from each of the five experimental groups at each sampling time. After the second blood sample, the fish were killed and the gonads were fixed in Bouin's solution. The technique of blood sampling is described in detail in Section III, above.

## V. Operations

Fish were anaesthetised in a 1:1000 ethyl-m-aminobenzoate solution until all opercular movements ceased and the fish were unable to maintain an upright position. Body weight was then recorded and the fish were wrapped in a wet paper towel. The operations were performed as follows:

*Blinding*

The fish was laid on its side on a wet paper towel. The membranes on the posterior side of the eye were grasped with fine notched tweezers and the membranes around the eye were cut. The eyeball was then slightly pulled out and blunt curved scissors were inserted from the posterior side of the eye, passing over the major blood vessel which is situated on the posterior side of the orbit. The optic nerve and the extrinsic eye muscles were cut and the eyeball removed. This procedure did not result in any visible bleeding. Fish were revived in oxygenated dechlorinated tap water. The healing process was rapid, and after about a day the fish were taking food.

*Pinealectomy*

The telencephalon was exposed using a technique developed and described by Peter (1970) and Peter and Gill (1975). Following this procedure, the saccus dorsalis and the pineal stalk, situated anterior to the optic lobes (Fig. 3), were visible under an operating microscope (24X). Gentle suction was applied with a Pasteur pipette attached to a vacuum system and the saccus dorsalis and the pineal stalk were sucked into the pipette. The operation was considered successful when the two structures were observed to move into the pipette and when some slight bleeding appeared at the region where the saccus dorsalis attaches to the brain. In order to remove the pineal body, gentle suction was applied to the inner surface of the cranium in the region anterior to the exposed area (Fig. 4).

The cranium was then filled with goldfish physiological saline solution and closed using the procedure described in Peter (1970) and Peter and Gill (1975). Stitches were removed without anaesthesia, three days after the operation.

*Sham operation*

All the steps described in the pinealectomy operation were performed except the following: after lifting the U-shaped bone flap, the pineal body, the pineal stalk and the saccus dorsalis were not removed. A sham blinding operation was not done.

## VI. Radioimmunoassay for Gonadotropin

All the serum samples were assayed with a radioimmunoassay specific for carp GTH (Crim *et al.*, 1976). This assay involves competition

Fig. 3. Diagrammatic representation of the dorsal view of the area of the brain exposed during pinealectomy.

d = diencephalon

ds = dorsal sac

o = optic lobes

p = pineal stalk

t = telencephalon

U = lifted U-shaped bone flap

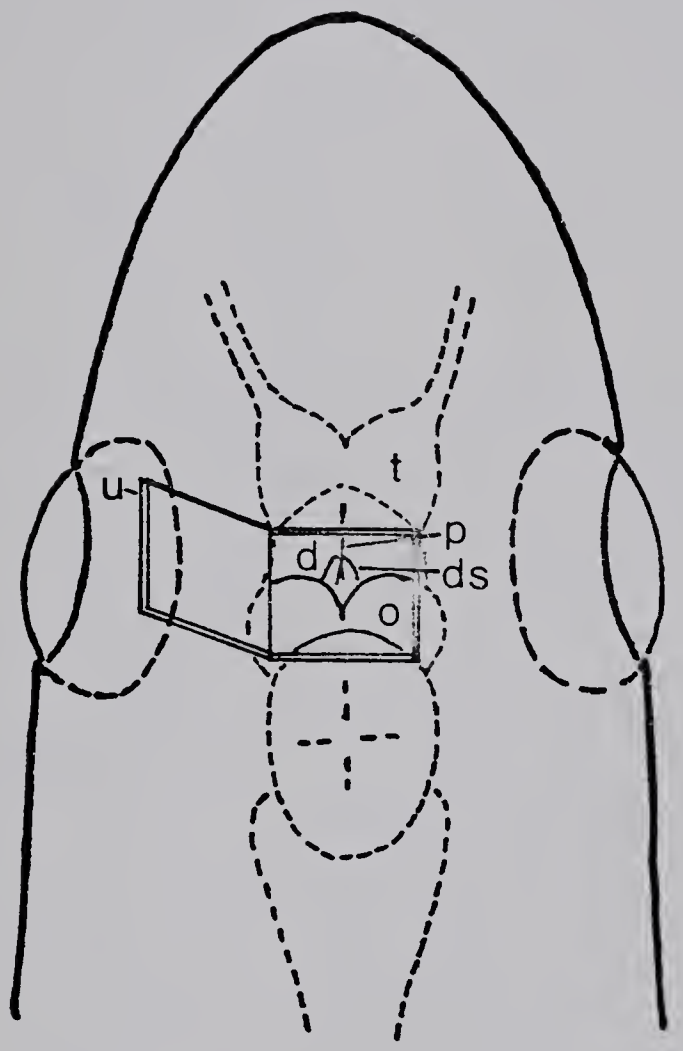

t
u
p
d
ds
o

Fig. 4. Diagrammatic representation of sagittal section through the pineal region of the goldfish, *Carassius auratus*.

c = cranium + dermis

ds = dorsal sac

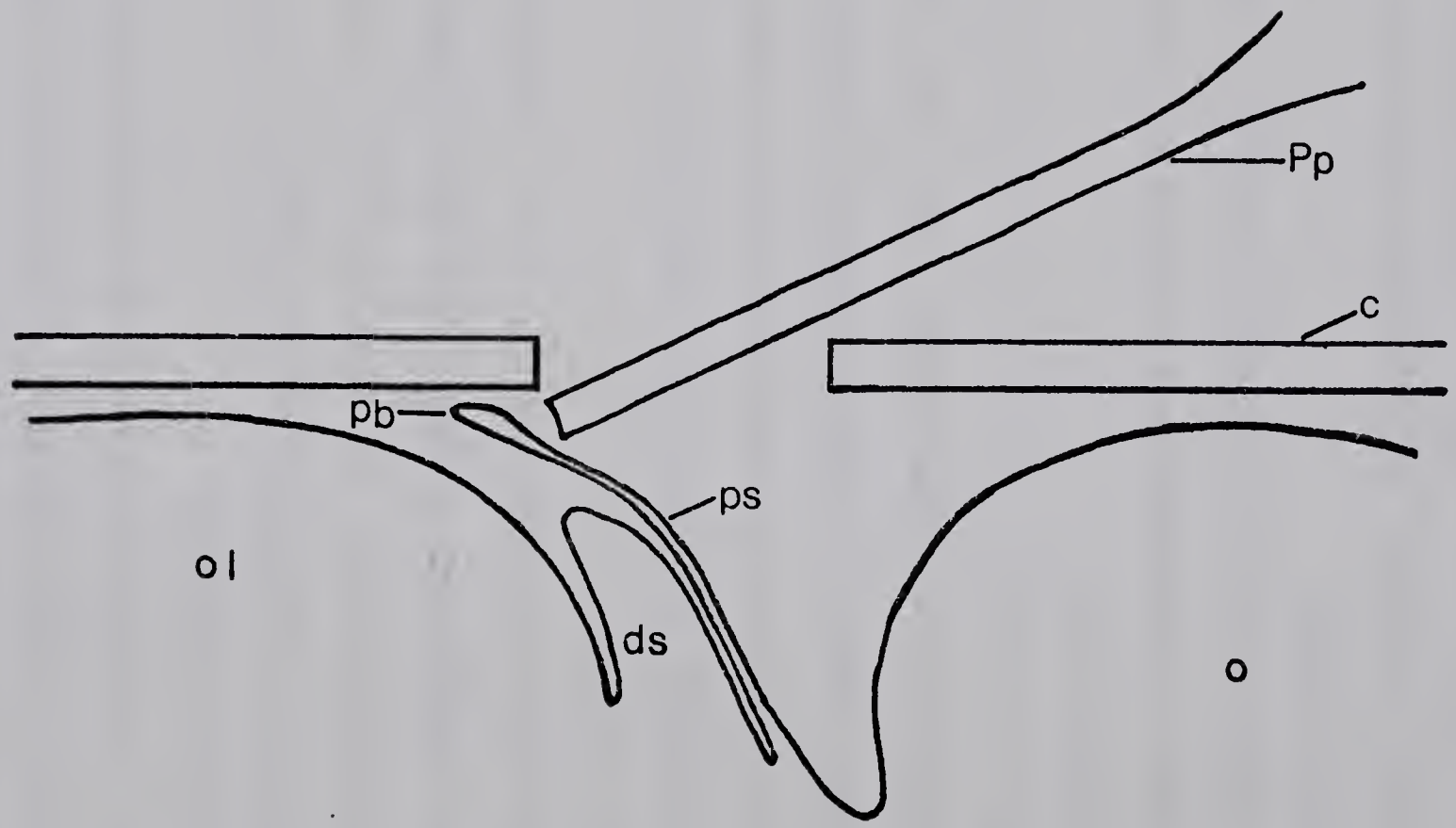
o = optic lobe

ol = olfactory lobe

pb = pineal body

Pp = Pasteur pipette

ps = pineal stalk


Pp
c
pb
ps
ds
ol
o

between the goldfish GTH in the serum samples and carp GTH labelled with $^{131}$I for binding sites on rabbit antibody raised to carp GTH. The antibody bound GTH was precipitated with a second antibody system using normal rabbit serum and an antibody raised in goat to rabbit serum. A similar assay has been developed by Breton *et al.* (1972) and several experiments validating this assay have been carried out in our laboratory (unpublished results).

*Within-assay variability* was determined as the standard deviation (S.D.) of values obtained from samples assayed several times in one RIA. The samples assayed were standard solutions of known concentrations of GTH: 0.25 ng/ml, 0.5 ng/ml and 1.0 ng/ml. The value of S.D.: $0.03 < S.D. < 0.08$.

*Between-assay variability* was determined as S.D. of values obtained for samples assayed in different RIAs and as a coefficient of correlation ($R^2$) of a series of samples assayed in two different RIAs. S.D. of values obtained for standard solutions of a known concentration ($\bar{x}_1 = 8.87$, $n = 5$ and $\bar{x}_2 = 2.04$, $n = 6$) were 2.69 and 0.41, respectively. The value of $R^2 = 0.8$.

*Mid-range of the assay* can be defined as the concentration of GTH/ml at which 50% of the radioactive hormone is displaced. Mid-range = 3.59 ± 0.53 ng GTH/ml.

*The least detectable concentration* is the concentration resulting in a response two standard deviations away from the zero dose response. The average least detectable concentration obtained for the RIAs of this study: 0.65 ± 0.26 ng GTH/ml of serum.

*Slope of the standard curve* = -1.80.

## VII. Histology

Following the standard wax embedding procedure and staining, the sections of gonads were observed under the microscope (X100) for the purpose of a qualitative and quantitative description. A slightly modified Yamazaki's (1965) classification of the cells found in the ovary at different stages of sexual maturity was used. The individual stages and the cells representative of these stages are described below.

*The first growth stage* is equivalent to the oogonial phase and chromatin nucleolus stage described by Yamazaki. It includes oogonia 12μ-14μ in diameter and small oocytes in the 12μ-20μ diameter range. Since it is technically difficult to count these small cells, a scale of three units (+ = few; ++ = moderate number; +++ = large number) was used to express the relative numbers of cells representative of this stage.

The next stage was designated as *peri-nucleolus stage*. Two types of oocytes are characteristic of this stage: oocytes in the early peri-nucleolus stage range from 20μ-50μ in diameter, the cytoplasm stains deeply with haemotoxylin and the nucleus contains large numbers of nucleoli. The oocytes in the late peri-nucleolus stage range from 110μ-160μ in diameter and the cytoplasm has a weak affinity for the haematoxylin.

In *the yolk vesicle stage*, oocytes 150μ-400μ in diameter are observed. They contain pale yolk vesicles either in the periphery of the cytoplasm or accumulating centripetally.

*Primary yolk stage* is the next phase recognised. Small yolk globules appear in the inner part of cytoplasm while yolk vesicles

occupy the outer two thirds of the cytoplasm. Oocytes have dimensions ranging from 350μ-900μ.

In *the secondary and tertiary yolk stage*, the oocytes reach their maximum size, the inner part of the cytoplasm contains yolk globules while yolk vesicles occupy few rows in the periphery of the cytoplasm.

The final stage recognised was *the atretic follicle stage*. Oocytes become irregular in shape, granulosa cells hypertrophy and invade the oocytes. Empty follicles are also classified as representing this stage. The three unit scale (+, ++, +++) is used to quantify this stage.

## VIII. Statistical Analysis

Statistical differences between groups in the daily rhythm experiment were determined by the Duncan multiple range test and in the pinealectomy-blinding experiment by Student's *t*-test for unpaired samples (Steel and Torrie, 1960). Student's *t*-test for paired samples was used to determine statistically significant differences between the presample values and 2000 hr sample values.

Differences were considered statistically significant when the p value was less than 0.05.

Computerized results of the Duncan multiple range test are expressed as follows:

20 4 8 24 12 16

where 20, 4, 8, 24, 12 and 16 represent the ranked values of serum GTH at 2000 hr, 0400 hr, 0800 hr, 2400 hr, 1200 hr and 1600 hr, respectively. Any two means underlined by the same line are not significantly

different while any two means not underlined by the same line are significantly different.

# RESULTS

## I. Daily Rhythm Experiment

*Fish with a regressed gonad*

The variations in the amount of serum GTH in fish with immature gonads in a quiescent state (also designated as regressed fish in this study), exposed to different conditions of photoperiod and temperature in September 1976, are shown in Figure 5. Individual sampling times from the three days during which the fish were blood sampled for the second time (see Fig. 1) were recombined to constitute a period of 24 hours. Each point represents the average of serum GTH from the fish sampled at each time. The data were analysed with the Duncan multiple range test; the computerized results of the Duncan's tests are presented in the caption of Figure 5. In the group subjected to the 16L:8D/21±1°C regime, the average value of serum GTH of fish sampled at 0400 hr was significantly higher than the value at 1600 hr. These results indicate that a peak in serum GTH was detected at 0400 hr. In the 8L:16D/21±1°C group, a peak in serum GTH level was found at 1200 hr. The value at 1200 hr was significantly higher than the values at 2400 hr and 0400 hr in this group. No statistically significant differences were found between the serum GTH values at different times of the 24 hour period in regressed fish exposed to the 16L:8D/12±1°C and the 8L:16D/12±1°C regimes.

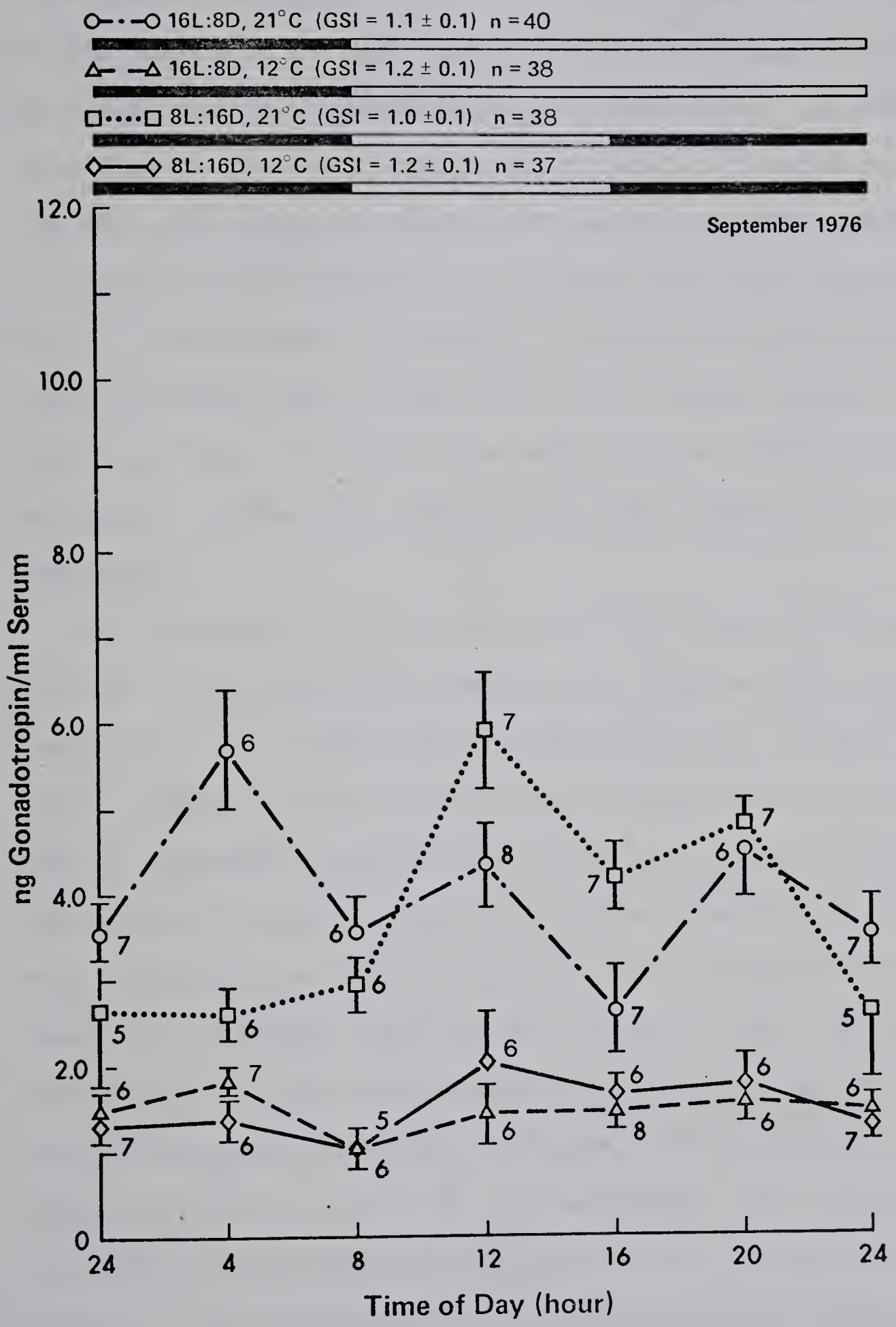
The differences between the means of the four experimental groups at each specific sampling time were also analysed using the Duncan multiple range test. At 0400 hr, the value from the 16L:8D/21±1°C

Fig. 5. Serum GTH levels (mean ± SEM) in a 24 hour period of fish with immature gonads in a quiescent state subjected to different conditions of photoperiod and temperature. The numbers beside each point indicate number of fish sampled at each time. The experimental photoperiod and temperature regime to which each group was exposed is indicated on top of the figure. The black horizontal bars represent the dark phase of the photoperiod, the empty bars represent the light phase. The numbers of fish used in each group and their average GSI (mean ± SEM) are also indicated on top of the figure.

Computerized results of Duncan multiple range test:
$p < 0.05$

| | |
|---|---|
| 16L:8D/21±1°C | 16 8 24 12 20 4 |
| 8L:16D/21±1°C | 4 24 8 16 20 12 |
| 16L:8D/12±1°C | 8 12 16 24 20 4 |
| 8L:16D/12±1°C | 8 24 4 16 20 12 |

| | | | | |
|---|---|---|---|---|
| 0400 hr | 8L/12°C | 16L/12°C | 8L/21°C | 16L/21°C |
| 0800 hr | 8L/12°C | 16L/12°C | 8L/21°C | 16L/21°C |
| 1200 hr | 16L/12°C | 8L/12°C | 16L/21°C | 8L/21°C |
| 1600 hr | 16L/12°C | 8L/12°C | 16L/21°C | 8L/21°C |
| 2000 hr | 16L/12°C | 8L/12°C | 16L/21°C | 8L/21°C |
| 2400 hr | 8L/12°C | 16L/12°C | 8L/21°C | 16L/21°C |


16L:8D, 21°C (GSI = 1.1 ± 0.1) n = 40
16L:8D, 12°C (GSI = 1.2 ± 0.1) n = 38
8L:16D, 21°C (GSI = 1.0 ±0.1) n = 38
8L:16D, 12°C (GSI = 1.2 ± 0.1) n = 37
September 1976
ng Gonadotropin/ml Serum
12.0
10.0
8.0
6.0
4.0
2.0
0
24
4
8
12
16
20
24
Time of Day (hour)

group was found to be significantly higher than the values from the other three groups. At 0800 hr and 2000 hr, the average values of serum GTH of fish exposed to the warm temperature were significantly higher than the values from fish exposed to the cold temperature. At 1200 hr, the value from the group subjected to the 8L:16D/21±1°C regime was higher than the values from the 16L:8D/12±1°C and 8L:16D/12±1°C groups. Also the values from the 16L:8D/21±1°C group was higher than the value from the 16L:8D/12±1°C group. At 1600 hr, the value from the 8L:16D/21±1°C group was higher than the values from the two groups exposed to the cold temperature. At 2400 hr, the value from the 16L:8D/21±1°C group was higher than the values from the two groups exposed to the cold temperature.

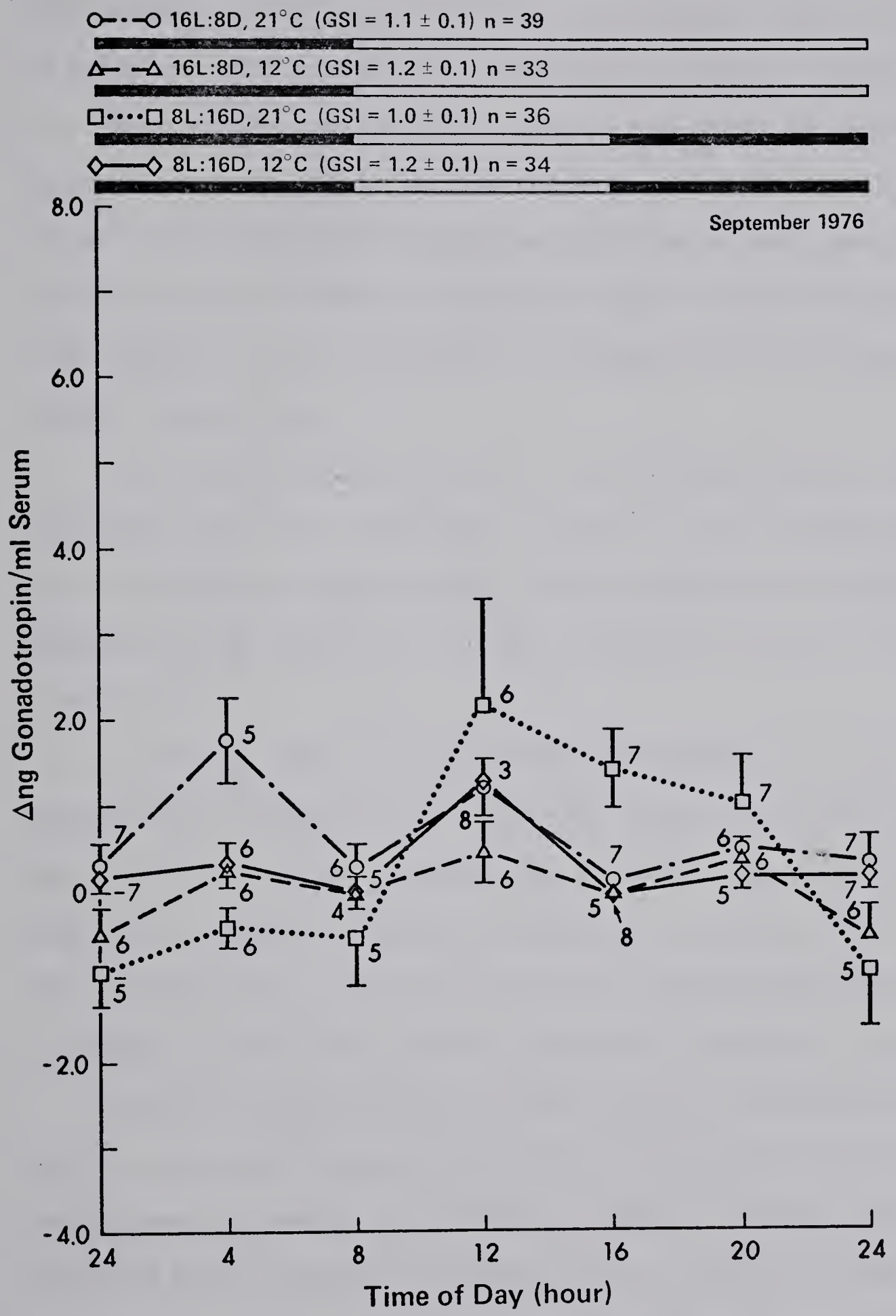
The variations in a 24 hour period in the amount of serum GTH at the time of the second blood sample of the regressed fish expressed as change from the presample taken at 2000 hr on day 4 (see Fig. 1) are shown in Figure 6. The presample value of serum GTH of a particular fish was subtracted from the value of the second sample for the same fish to give the change, which can be either a positive or a negative value. Each point on Figure 6 represents the average value of the change for the fishes sampled at that particular time. The values have been analysed with the Duncan multiple range test in the same way as the absolute values represented in Figure 5. For the purposes of computer analysis a constant (k = 10) was added to all the values. The results of the computer analysis are shown in the caption for Figure 6. In the group subjected to the 8L:16D/21±1°C regime, the average value of the change in serum GTH of the fish sampled at

Fig. 6. Serum GTH levels (mean ± SEM) in a 24 hour period of fish with immature gonads in a quiescent state, expressed as changes from the presample taken on day 4. Numbers beside each point indicate number of fish sampled at each time. Experimental photoperiod and temperature regimes are indicated on top of the figure. Number of fish used in each group and their average GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | |
|---|---|
| 16L:8D/21±1°C | 16 8 24 20 4 12 |
| 8L:16D/21±1°C | 24 4 8 20 16 12 |
| 16L:8D/12±1°C | 24 12 4 16 20 8 |
| 8L:16D/12±1°C | 16 12 24 4 20 8 |


16L:8D, 21°C (GSI = 1.1 ± 0.1) n = 39
16L:8D, 12°C (GSI = 1.2 ± 0.1) n = 33
8L:16D, 21°C (GSI = 1.0 ± 0.1) n = 36
8L:16D, 12°C (GSI = 1.2 ± 0.1) n = 34
September 1976
Δng Gonadotropin/ml Serum
8.0
6.0
4.0
2.0
0
-2.0
-4.0
24
4
8
12
16
20
24
Time of Day (hour)

1200 hr was found to be significantly higher than the values from the fish sampled at 2400 hr and 0400 hr, respectively. Also the mean value of change at 1600 hr was higher than the mean value of change at 2400 hr. These results suggest that a peak in the amount of serum GTH occurred at about 1200 hr in fish subjected to the 8L:16D/21±1°C regime. No statistically significant differences were found between the mean values of change at different times of the 24 hour period in fish subjected to the 16L:8D/21±1°C, 16L:8D/12±1°C or 8L:16D/12±1°C regimes, respectively.

Using a paired Student's *t*-test, the absolute values of serum GTH from fish sampled the second time at 2000 hr on day 7 were compared with the presample values on day 4 of the same fish. No significant differences were found in any of the four groups of fish in the experiment.

The average GSIs of the regressed fish subjected to the 16L:8D/21±1°C, 8L:16D/21±1°C, 16L:8D/12±1°C and 8L:16D/12±1°C regimes were 1.1±0.1%, 1.0±0.1%, 1.2±0.1% and 1.2±0.1%, respectively; the proportion of males identified in each group were 27%, 21%, 10% and 16%, respectively. A Student's *t*-test for unpaired values was used to compare the GSI values and no significant differences were found.

Histological examination of gonads from the regressed fish showed that all the gonads examined contained elements representative of the early stages of gonadal maturation. As Table 1 indicates, the ovaries contained mostly yolkless components such as oogonia and small oocytes characteristic of the chromatin nucleus stage and oocytes in the perinucleus stage (see pp. 26 and 27 for detailed description of each

TABLE 1. Numerical evaluation of cells found in the gonads of regressed female fish. (See pp. 26 and 27 for detailed description of each stage.)

| Experimental group | GSI ($\bar{x}$ ± SEM) | Number of cells/mm$^2$ of gonad ($\bar{x}$ ± SEM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | First growth stage | Perinucleolus stage | Yolk vesicle stage | Primary yolk stage | Secondary and tertiary yolk stage | Atretic follicle stage |
| 16L:8D/21°C | 1.1±0.1% ($n_1$=40) | ++ ($n_2$=6) | 30.0±11.6 | 3.41±1.1 | 0.58±0.5 | 0.08±0 | — |
| 8L:16D/21°C | 1.0±0.1% ($n_1$=38) | ++ ($n_2$=10) | 46.4±10.6 | 2.74±1.2 | 0.05±0 | 0.15±0.14 | — |
| 16L:8D/12°C | 1.2±0.1% ($n_1$= 38) | ++ ($n_2$=3) | 57.0±16.0 | 2.83±1.47 | 0 | 0 | — |
| 8L:16D/12°C | 1.2±0.1% ($n_1$=37) | ++ ($n_2$=14) | 32.6±8.38 | 1.53±0.45 | 0.28±0.2 | 0.5±0.38 | — |

stage). Some oocytes in the yolk vesicle stage were observed. Only 4 ovaries from the total of 44 ovaries examined (9%) contained oocytes in the 1°, 2° or 3° yolk stages. The proportions of the various cell types were observed to be similar in the ovaries of animals examined from the four experimental groups. Figure 7 represents a typical ovary from a regressed female fish. Ahsan's (1966b) description of gonads in male golden shiner was used to identify immature testis.

*Females undergoing ovarian recrudescence*

The variations in the amount of serum GTH over a 24 hr period in female fish undergoing ovarian recrudescence (also designated as maturing fish, January 1977), are shown in Figure 8. Computerized results of the Duncan multiple range test are shown in the caption for Figure 8. In the group exposed to the 16L:8D/21±1°C regime, the average value of serum GTH of fish sampled at 1200 hr was found to be significantly higher than the values at 0400 hr and 2400 hr. This suggests that under the 16L:8D/21±1°C regime, a peak in serum GTH occurred at about 1200 hr. No differences were found between values at the different times of the 24 hour period in fish subjected to the 8L:16D/21±1°C or the 8L:16D/12±1°C regime, respectively. In the group of fish subjected to 16L:8D/12±1°C, a peak of serum GTH occurred at about 1600 hr as indicated by the fact that the value of serum GTH at 1600 hr was found to be higher than the values at 0800 hr, 1200 hr, 2000 hr and 2400 hr.

The means of the four experimental groups at each sampling time were also compared. At 1200 hr, 1600 hr and 2000 hr, the values of the

Fig. 7. Cross-section through the gonad of a regressed female fish (X260)

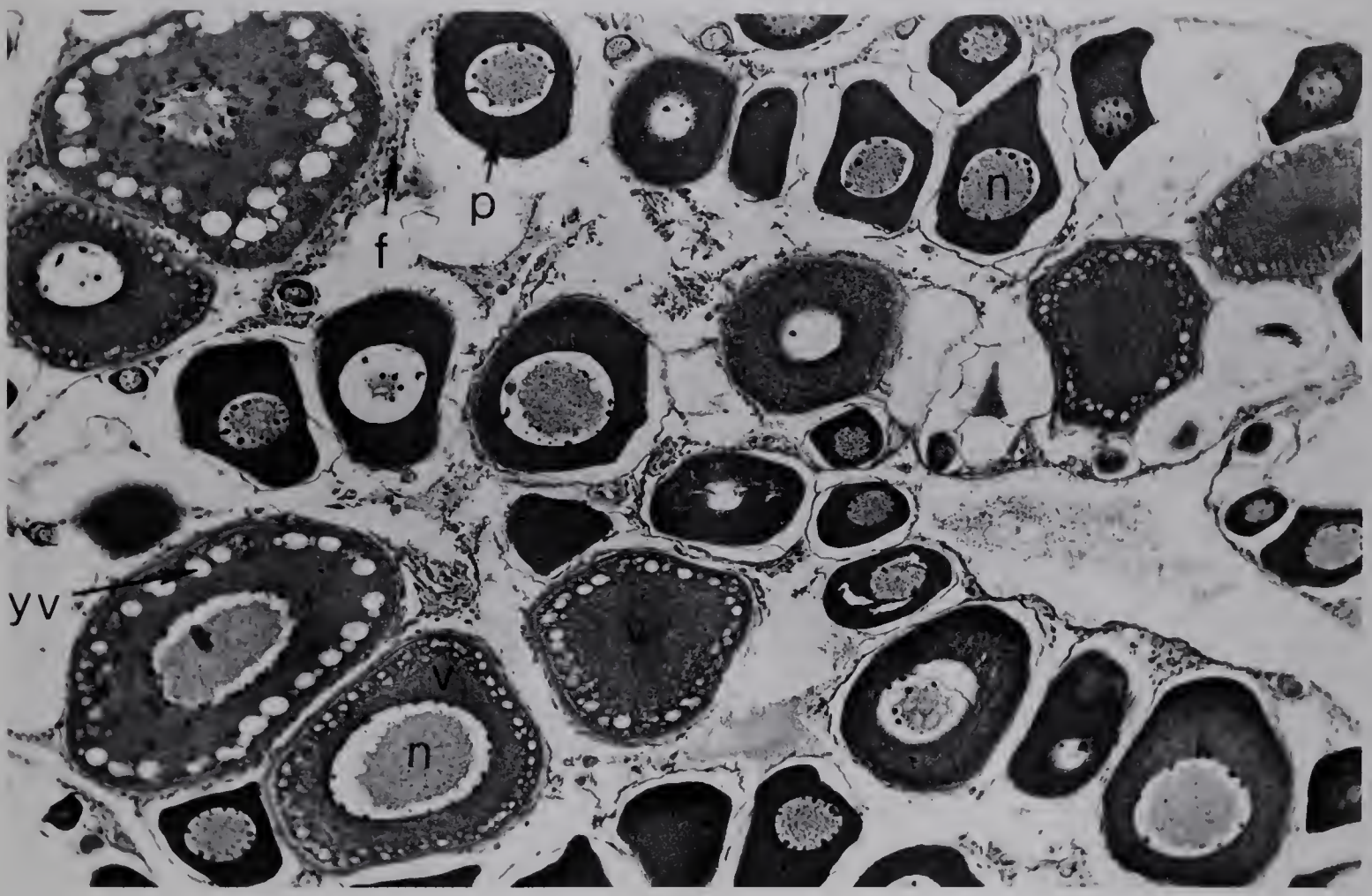
f = oogonia and oocytes in the first growth stage

n = nucleus

p = oocytes in the perinucleolus stage

v = oocytes in the yolk vesicle stage

yv = yolk vesicles


p
f
n
yv
v
n

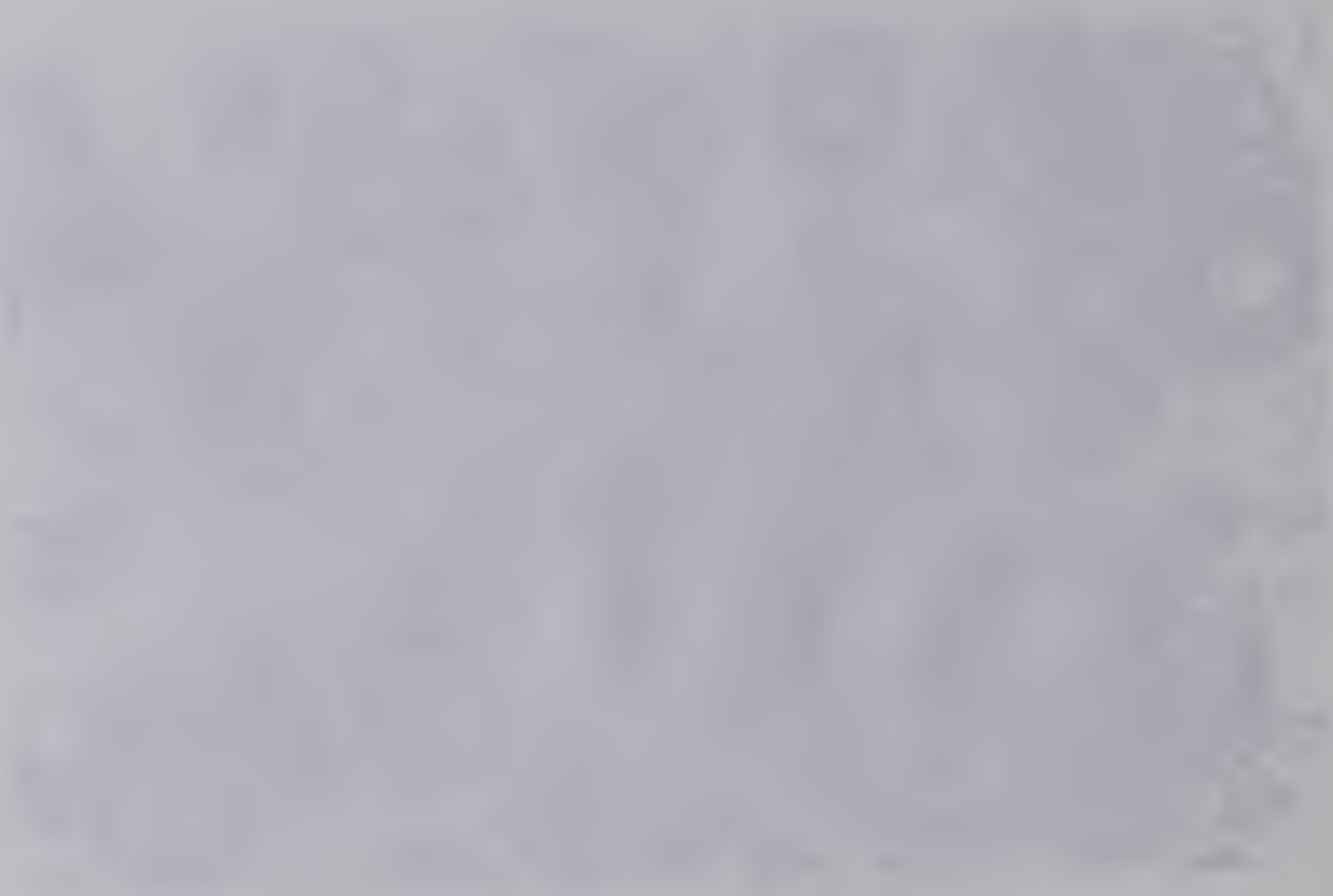


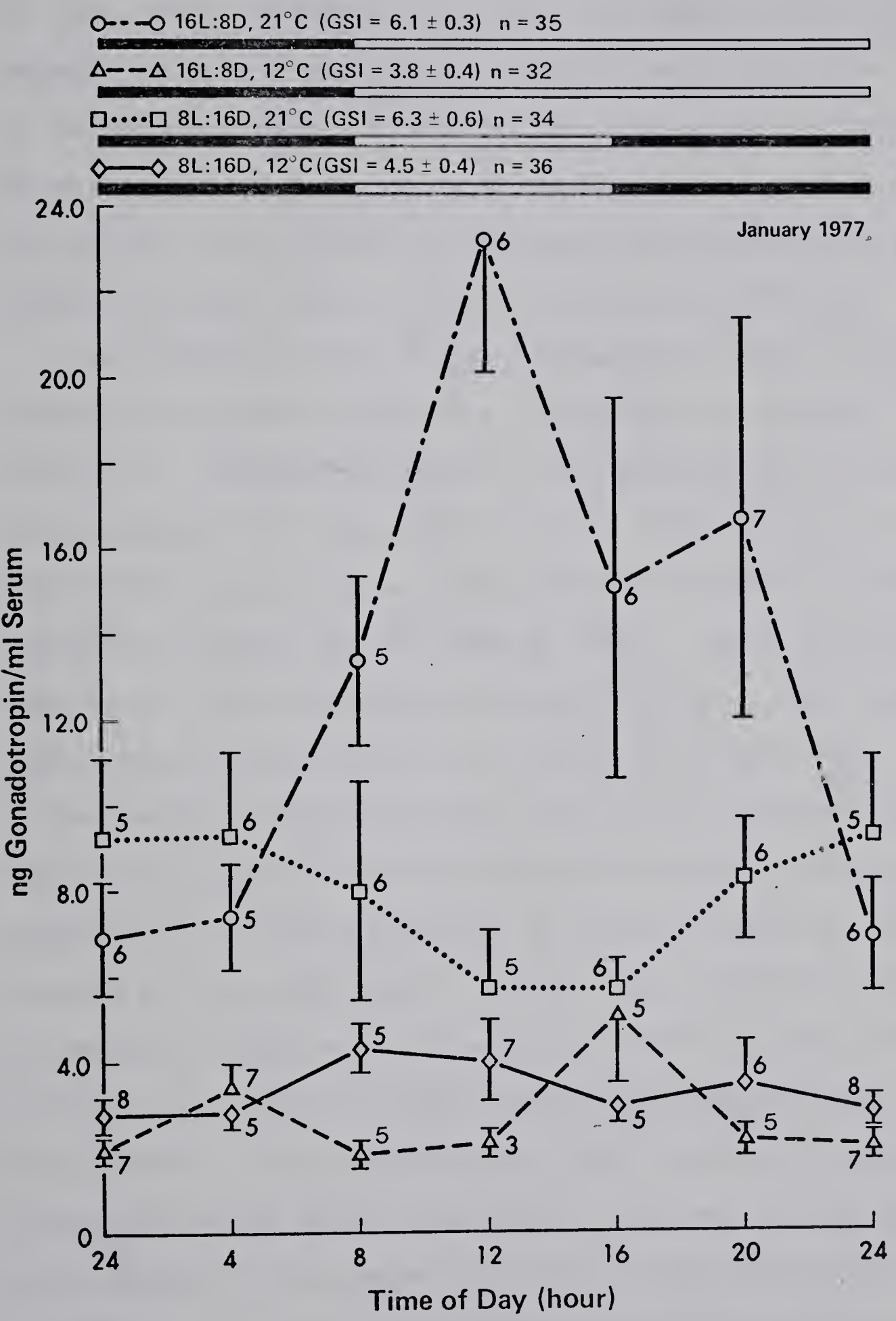
Fig. 8. Serum GTH levels (mean ± SEM) in a 24 hour period of female fish undergoing ovarian recrudescence, subjected to different conditions of photoperiod and temperature. Numbers beside each point indicate number of fish sampled at each time. Experimental photoperiod and temperature regimes are indicated on top of the figure. Number of fish in each group and their GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | |
|---|---|
| 16L:8D/21±1°C | 24 4 8 16 20 12 |
| 8L:16D/21±1°C | 12 16 8 20 24 4 |
| 16L:8D/12±1°C | 8 24 12 20 4 16 |
| 8L:16D/12±1°C | 12 16 24 4 20 8 |

| | | | | |
|---|---|---|---|---|
| 0400 hr | 8L/12°C | 16L/12°C | 8L/21°C | 16L/21°C |
| 0800 hr | 16L/12°C | 8L/12°C | 8L/21°C | 16L/21°C |
| 1200 hr | 16L/12°C | 8L/12°C | 8L/21°C | 16L/21°C |
| 1600 hr | 8L/12°C | 16L/12°C | 8L/21°C | 16L/21°C |
| 2000 hr | 16L/12°C | 8L/12°C | 8L/21°C | 16L/21°C |
| 2400 hr | 16L/12°C | 8L/12°C | 16L/21°C | 8L/21°C |


16L:8D, 21°C (GSI = 6.1 ± 0.3) n = 35
16L:8D, 12°C (GSI = 3.8 ± 0.4) n = 32
8L:16D, 21°C (GSI = 6.3 ± 0.6) n = 34
8L:16D, 12°C (GSI = 4.5 ± 0.4) n = 36
January 1977
ng Gonadotropin/ml Serum
24.0
20.0
16.0
12.0
8.0
4.0
0
24
4
8
12
16
20
24
Time of Day (hour)

16L:8D/21±1°C group were higher than the values of the other three groups at these times. At 0400 hr and 2400 hr, the means of the two groups exposed to the warm temperature were significantly higher than the values of the two cold groups. At 0800 hr, the value of the 16L:8D/21±1°C group was higher than the values of the two groups exposed to the cold temperature. Also at 0800 hr, the value of the 8L:16D/21±1°C group was found to be higher than the value of the 16L:8D/12±1°C group.

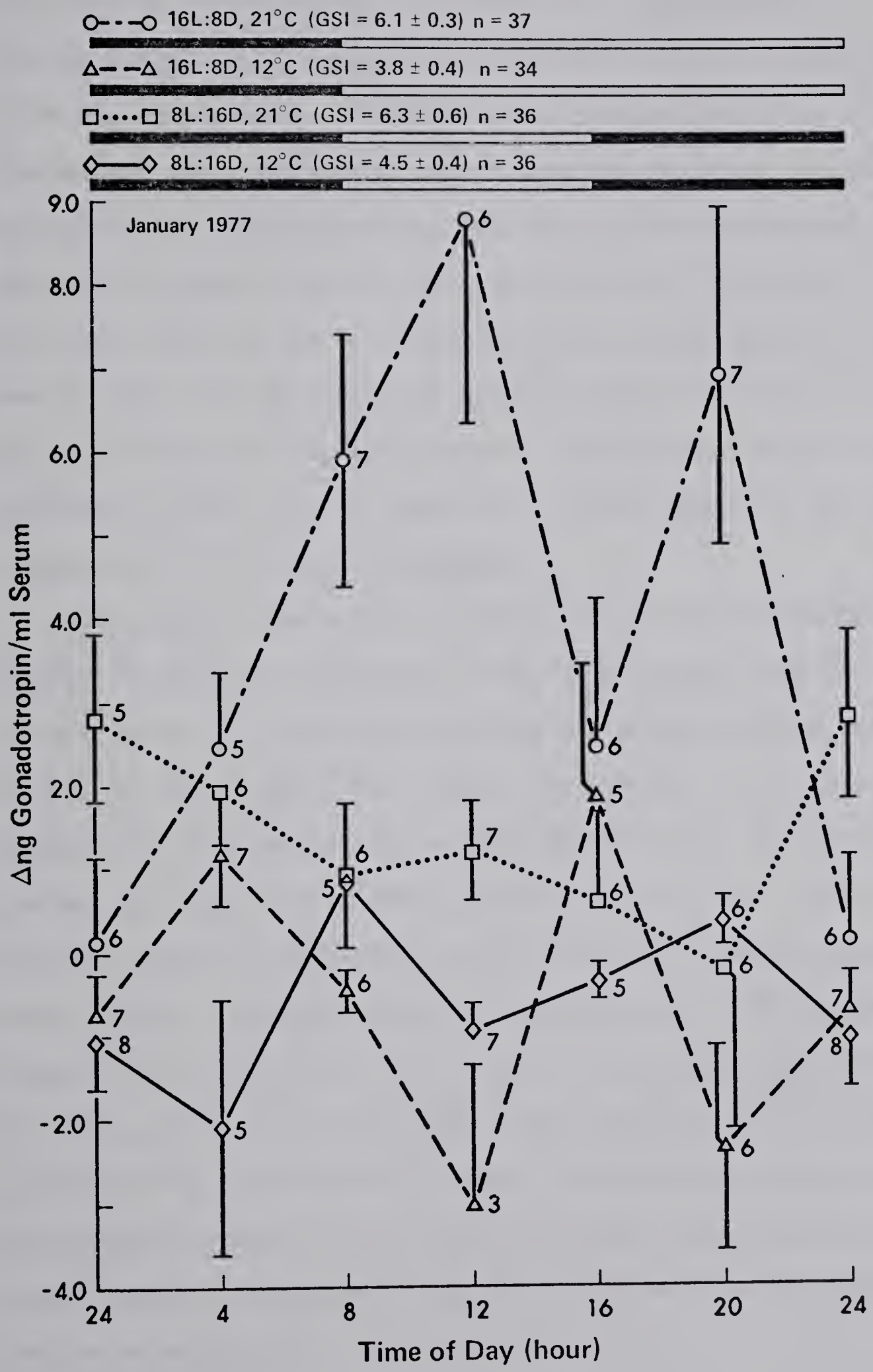
The variations in the amount of serum GTH in females undergoing ovarian recrudescence, expressed as change from the presample are shown in Figure 9. Computerized results of the Duncan multiple range tests are presented in the caption for Figure 9. In the group exposed to the 16L:8D/21±1°C regime, the mean value from fish sampled at 1200 hr was significantly higher than the value at 2400 hr. These results indicate that a peak of serum GTH occurred at about 1200 hr. There were no significant differences between the values at different times of the 24 hour period in the 8L:16D/21±1°C group. In the 16L:8D/12±1°C group, the average serum GTH values at 1200 hr and 2400 hr were found to be significantly lower than the values at 0400 hr and 1600 hr. Two peaks, situated at 0400 hr and 1600 hr, respectively, occurred in this group. In the group of fish subjected to the 8L:16D/12±1°C regime, the value at 0400 hr was found to be significantly lower than the values at 0800 hr and 2000 hr. This indicates that a peak in serum GTH occurred in the time period between 0800 hr and 2000 hr, inclusive, in this group. A paired Student's *t*-test showed that the presample values and the values at 2000 hr on day 7 were not significantly different for the various groups, except for the group subjected to the 16L:8D/21±1°C regime.

Fig. 9. Serum GTH levels expressed as changes from the presample (mean ± SEM) in a 24 hour period of female undergoing ovarian recrudescence, subjected to different photoperiod and temperature regimes. Numbers beside each point indicate number of fish sampled at each time. Experimental photoperiod and temperature regimes are indicated on top of the figure. Numbers of fish used in each group and their GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | |
|---|---|
| 16L:8D/21±1°C | 24 4 20 16 8 12 |
| 8L:16D/21±1°C | 16 8 12 20 4 24 |
| 16L:8D/12±1°C | 12 20 24 8 4 16 |
| 8L:16D/12±1°C | 4 24 12 16 20 8 |


16L:8D, 21°C (GSI = 6.1 ± 0.3) n = 37
16L:8D, 12°C (GSI = 3.8 ± 0.4) n = 34
8L:16D, 21°C (GSI = 6.3 ± 0.6) n = 36
8L:16D, 12°C (GSI = 4.5 ± 0.4) n = 36
January 1977
Δng Gonadotropin/ml Serum
9.0
8.0
6.0
4.0
2.0
0
-2.0
-4.0
24
4
8
12
16
20
24
Time of Day (hour)

Average GSIs of female fish undergoing ovarian recrudescence, subjected to the 16L:8D/21±1°C, 8L:16D/21±1°C, 16L:8D/12±1°C and the 8L:16D/12±1°C regimes were 6.1±0.3%, 6.3±0.6%, 3.8±0.4% and 4.5±0.4%, respectively. The GSI of each group subjected to the warm temperature was significantly higher than the GSI of the two groups exposed to the cold temperature, regardless of the experimental photoperiod regime. Specifically, the GSI of the 16L:8D/21±1°C group was higher than the GSI of the 16L:8D/12±1°C and 8L:16D/12±1°C groups, and the GSI of the 8L:16D/21±1°C group was higher than the GSI of the 16L:8D/12±1°C and 8L:16D/12±1°C groups. There was no significant difference between GSIs of experimental groups exposed to the same temperature but different photoperiods.

Histological examination of ovaries from female fish undergoing ovarian recrudescence showed that 100% of the gonads examined contained oocytes in the 1° yolk stage and 49% of the gonads examined contained oocytes in the 2° and 3° yolk stage. The ovaries also contained oogonia and small oocytes in the first growth stage, oocytes in the perinucleus stage and the yolk vesicle stage (Table 2). Atretic follicles were not observed in ovaries from any of the four experimental groups. The numbers of cells representing the first growth stage, yolk vesicle stage, 1°, 2° and 3° yolk stage seemed similar in all four groups. The ovaries from fish exposed to the cold temperature (16L:8D/12±1°C, 8L:16D/12±1°C) seemed to contain more oocytes in the perinucleolus stage than the ovaries from fish subjected to warm temperature. Figure 10 represents a typical ovary from a female undergoing ovarian recrudescence.

TABLE 2. Numerical evaluation of cells found in the gonads of female fish undergoing ovarian recrudescence.

(See pp. 37 and 42 for detailed description of each stage.)

| | | Number of cell/mm$^2$ of gonad ($\bar{x}$ ± SEM) | | | | | |
|---|---|---|---|---|---|---|---|
| Experimental group | GSI ($\bar{x}$ ± SEM) | First growth stage | Perinucleolus stage | Yolk vesicle stage | Primary yolk stage | Secondary and tertiary yolk stage | Atretic follicle stage |
| 16L:8D/21°C | 6.1±0.3 ($n_1$=35) | + ($n_2$=12) | 20.2±5.4 | 3.94±1.2 | 5.4±0.9 | 0.8±0.3 | – |
| 8L:16D/21°C | 6.3±0.6 ($n_1$=34) | + ($n_2$=11) | 20.4±4.8 | 3.9±0.8 | 6.42±0.9 | 0.4±0.2 | – |
| 16L:8D/12°C | 3.8±0.4 ($n_1$=32) | + ($n_2$=8) | 29.8±12.5 | 3.3±0.5 | 6.4±1.1 | 0.4±0.2 | – |
| 8L:16D/12°C | 4.5±0.4 ($n_1$=36) | + ($n_2$=8) | 33.5±9.4 | 5.2±1.1 | 5.4±1.1 | 0.73±0.5 | – |

$N_2$ = total number of fish in the group.

$N_1$ = number of ovaries examined in the group.

Fig. 10. Cross-section through the gonad of a female fish undergoing overian recrudescence. (X150)

f = oogonia and oocytes in the first growth stage

n = nucleus

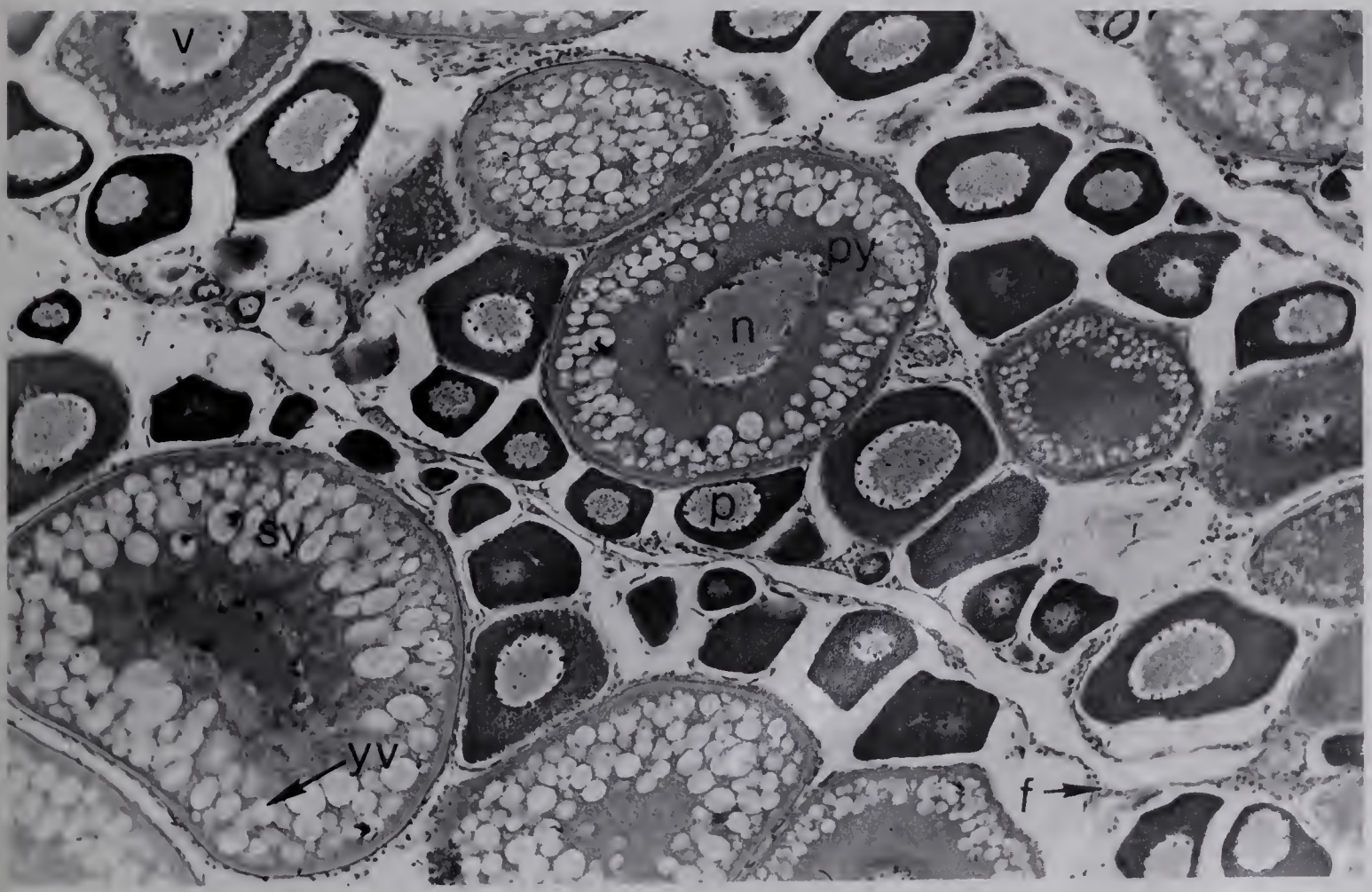
p = oocyte in the perinucleolus stage

py = oocyte in the 1°yolk stage

sy = oocyte in the 2°yolk stage

v = oocyte in the yolk vesicle stage

yv = yolk vesicles


v
py
n
p
sy
yv
f

*Females with a mature ovary*

The variations in the amount of serum GTH over a 24 hour period, in female fish with a mature ovary (March 1976) exposed to different conditions of temperature and photoperiod, are shown in Figure 11. Computerized results of the Duncan multiple range tests are shown in the caption for Figure 11. In the group exposed to the 16L:8D/21±1°C regime, the value of serum GTH of fish sampled at 1200 hr was found to be significantly higher than the values at 1600 hr, 2000 hr and 2400 hr. This indicates that a peak in serum GTH occurred at about 1200 hr. There were no significant differences between the values of serum GTH at different times of the 24 hour period in fish subjected to the 8L:16D/21±1°C regime. In the 16L:8D/12±1°C group, the average value of serum GTH of the fish sampled at 2000 hr was significantly higher than the value at 0800 hr. Therefore a peak in serum GTH was detected at 2000 hr in this group. In the group subjected to the 8L:16D/12±1°C regime, the values at 0800 hr and 2000 hr were found to be significantly higher than the values at 1200 hr and 1600 hr, and the value at 2000 hr was higher than the value at 2400 hr. This indicates that two peaks of serum GTH occurred under this regime, at 0800 hr and 2000 hr, respectively.

The means of the four experimental groups at each individual sampling time were compared. At 0400 hr, 1200 hr and 1600 hr, the values of serum GTH of fish subjected to the warm temperature were significantly higher than the values from the two cold groups. At 0800 hr, 2000 hr and 2400 hr, no significant differences between the four means were found.

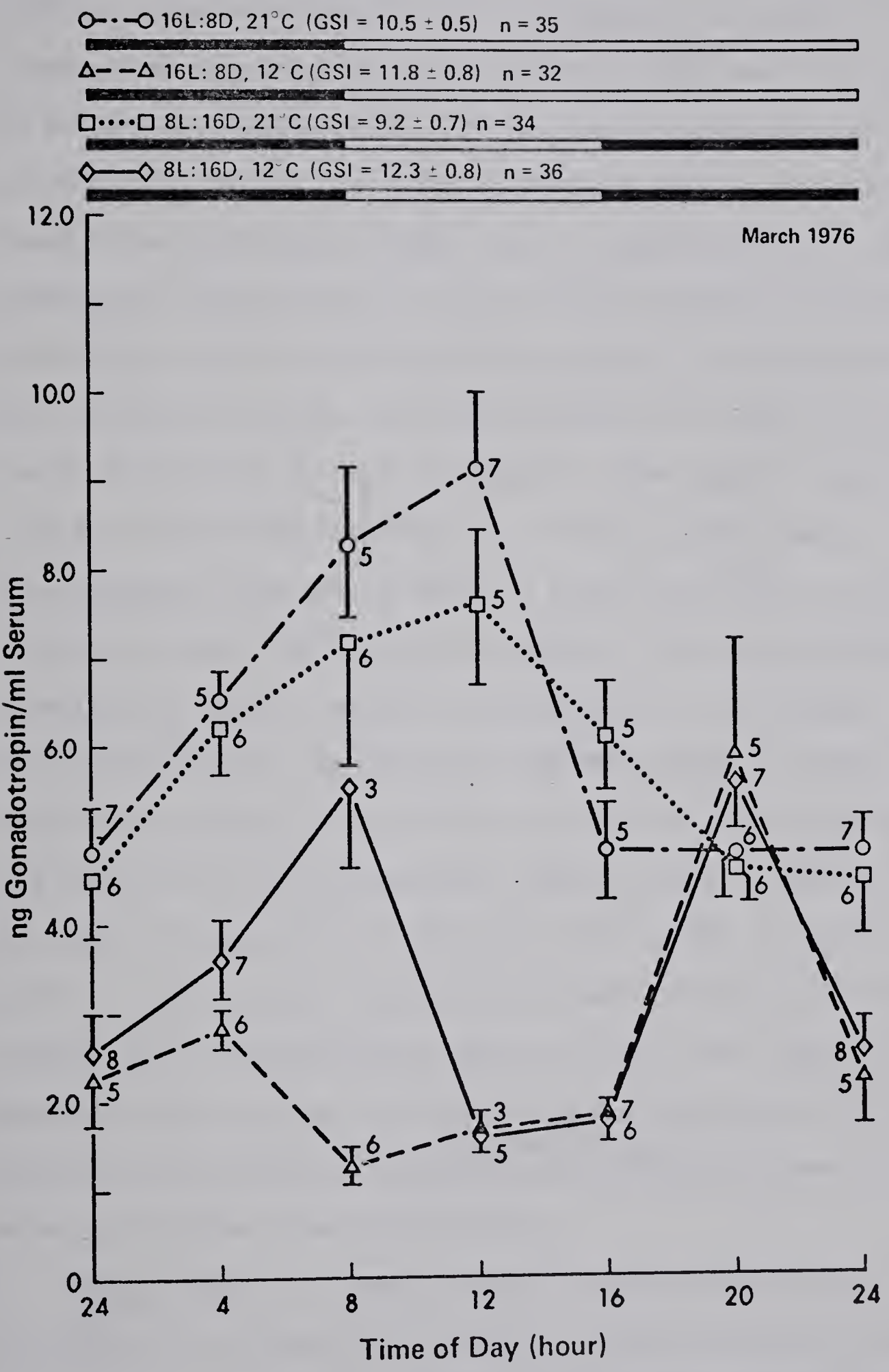
Fig. 11. Serum GTH levels (mean ± SEM) in a 24 hour period of female fish with a mature ovary, subjected to different conditions of photoperiod and temperature. Numbers beside each point indicate the number of fish sampled at each time. Experimental photoperiod and temperature regimes are indicated on top of the figure. Number of fish in each group and their GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | |
|---|---|
| 16L:8D/21±1°C | 20 24 16 4 8 12 |
| 8L:16D/21±1°C | 24 20 16 4 8 12 |
| 16L:8D/12±1°C | 8 12 16 24 4 20 |
| 8L:16D/12±1°C | 12 16 24 4 8 20 |

| | |
|---|---|
| 0400 hr | 16L/12°C 8L/12°C 8L/21°C 16L/21°C |
| 0800 hr | 16L/12°C 8L/12°C 8L/21°C 16L/21°C |
| 1200 hr | 8L/12°C 16L/12°C 8L/21°C 16L/21°C |
| 1600 hr | 8L/12°C 16L/12°C 16L/21°C 8L/21°C |
| 2000 hr | 8L/21°C 16L/21°C 8L/12°C 16L/12°C |
| 2400 hr | 16L/12°C 8L/12°C 8L/21°C 16L/21°C |


16L:8D, 21°C (GSI = 10.5 ± 0.5) n = 35
16L: 8D, 12°C (GSI = 11.8 ± 0.8) n = 32
8L:16D, 21°C (GSI = 9.2 ± 0.7) n = 34
8L:16D, 12°C (GSI = 12.3 ± 0.8) n = 36
March 1976
ng Gonadotropin/ml Serum
12.0
10.0
8.0
6.0
4.0
2.0
0
24
4
8
12
16
20
24
Time of Day (hour)

The variations in the amount of serum GTH of the fish with mature ovaries, expressed as change from the presample, are shown in Figure 12. Computerized results of the Duncan multiple range tests are presented in the caption for Figure 12. In the group of fish subjected to the 16L:8D/21±1°C regime, the values at 0400 hr, 0800 hr and 1200 hr were found to be significantly higher than the value at 1600 hr. Consequently the highest levels of serum GTH were detected in the time period from 0400 hr to 1200 hr in fish exposed to 16L:8D/21±1°C. In the 8L:16D/21±1°C group, the value at 0800 hr was found to be significantly higher than the value at 2400 hr. These results suggest that a peak of serum GTH occurred at about 0800 hr in this group. In the group exposed to the 16L:8D/12±1°C, a significant peak of serum GTH occurred at about 2000 hr since the value at 2000 hr was found to be significantly higher than all the other values in this group. In the 8L:16D/12±1°C group, the value of serum GTH at 0800 was found to be significantly higher than the values at 1200 hr, 1600 hr and 2400 hr, and the value at 2000 hr was higher than the value at 2400 hr. Thus, two peaks of serum GTH were detected in this group, at 0800 hr and 2000 hr, respectively. The absolute values of serum GTH from fish sampled for the second time at 2000 hr on day 7 were compared to the presample values of the same fish, using the paired Student's *t*-test. There were no statistically significant differences between the means of any of the four experimental groups.

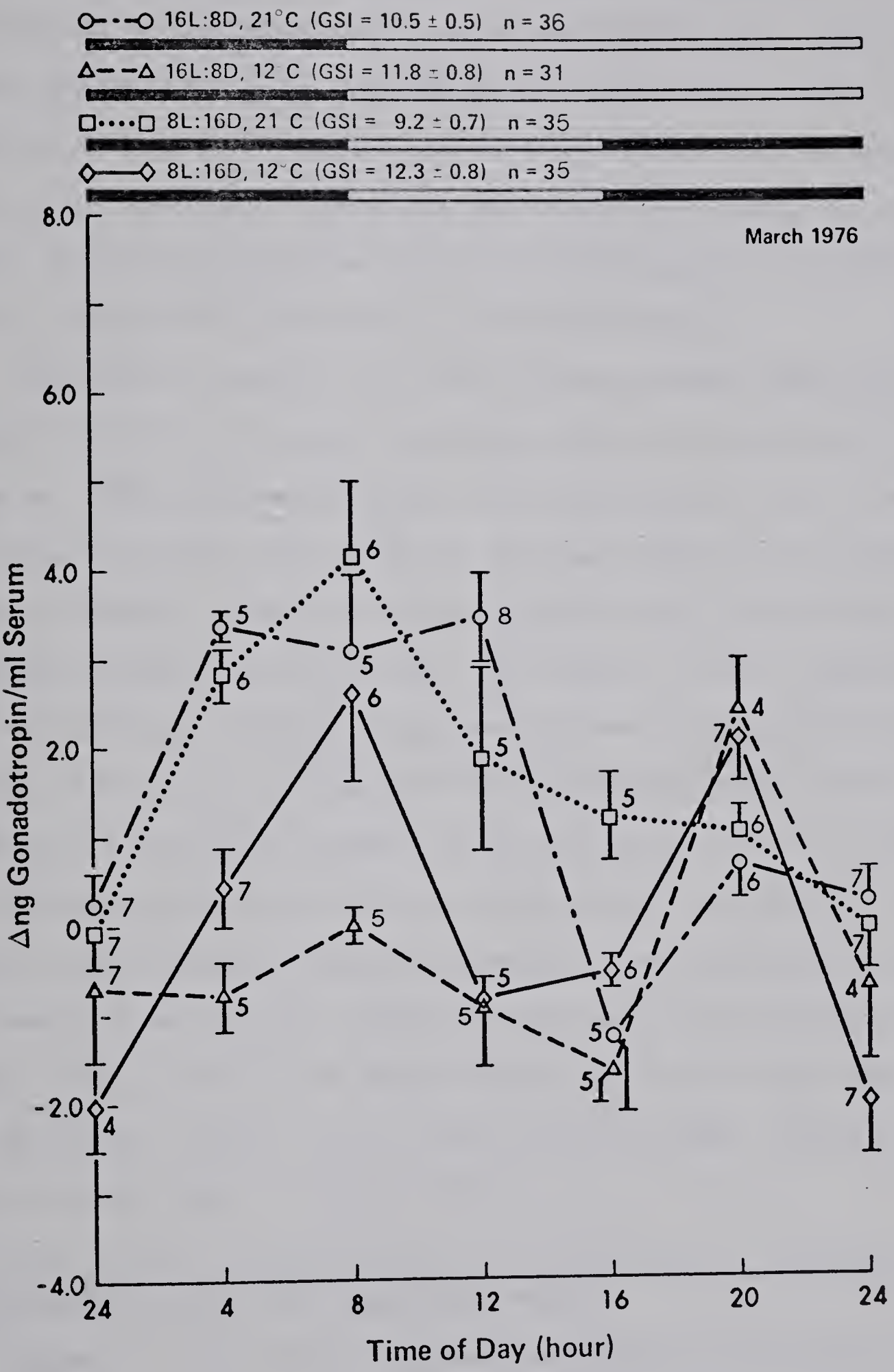
The average GSI of mature female fish subjected to the 16L:8D/21±1°C, 8L:16D/21±1°C, 16L:8D/12±1°C and 8L:16D/12±1°C regimes were 10.5±0.5%, 9.2±0.7%, 11.8±0.8% and 12.3±0.8%, respectively.

Fig. 12. Serum GTH levels expressed as changes from presample (mean ± SEM) of female fish with a mature ovary, subjected to different conditions of photoperiod and temperature. Numbers beside each point indicate number of fish sampled at each time. Experimental photoperiod and temperature regimes are indicated on top of the figure. Number of fish used in each group and their GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | |
|---|---|
| 16L:8D/21±1°C | 16 24 20 8 12 4 |
| 8L:16D/21±1°C | 24 20 16 12 4 8 |
| 16L:8D/12±1°C | 16 12 4 24 8 20 |
| 8L:16D/12±1°C | 24 12 16 4 20 8 |


16L:8D, 21°C (GSI = 10.5 ± 0.5) n = 36
16L:8D, 12°C (GSI = 11.8 ± 0.8) n = 31
8L:16D, 21°C (GSI = 9.2 ± 0.7) n = 35
8L:16D, 12°C (GSI = 12.3 ± 0.8) n = 35
March 1976
8.0
6.0
4.0
2.0
0
-2.0
-4.0
Δng Gonadotropin/ml Serum
24
4
8
12
16
20
24
Time of Day (hour)

The GSI of the 8L:16D/12±1°C group was found to be significantly higher than the GSI of the 16L:8D/21±1°C and 8L:16D/21±1°C groups. The GSI of the 16L:8D/12±1°C group was significantly higher than the GSI of the 8L:16D/21±1°C group. No significant differences were found between the GSIs of the 8L:16D/12±1°C group and the 16L:8D/12±1°C group, the GSIs of the 16L:8D/21±1°C group and the 8L:16D/21±1°C group, and the GSIs of the 16L:8D/12±1°C group and the 16L:8D/21±1°C group.

Histological examination of ovaries from the mature female fish showed that 100% of the gonads examined contained oocytes in the 1° yolk stage and 90% of the gonads examined contained oocytes in the 2° and 3° yolk stage. All the gonads (Table 3) also had oocytes in the perinucleus stage and oocytes in the yolk vesicle stage (see pp. 26 and 27 for detailed description of each stage). The numbers of cells representative of all these individual stages were observed to be similar in the ovaries of fish examined from the four experimental groups. On the average, all the ovaries, except ovaries from the 16L:8D/21±1°C groups, also contained elements of the first growth stage (see Table 3). Cells in the atretic follicle stage were observed in the 16L:8D/21±1°C group and even more cells in this stage were observed in the 8L:16D/21±1°C group. Ovaries from the two groups exposed to the cold temperature did not contain any cells in the atretic follicle stage. Figure 13 shows a typical ovary of a mature fish.

*Fish with gonads at different stages of gonadal maturity, subjected to the same photoperiod and temperature regime*

Figures 14, 15, 16 and 17 represent variations in the amount of serum GTH over a 24 hour period in fish with immature gonads in a

TABLE 3. Numerical evaluation of cells found in the gonads of mature female fish. (See pp. 49-55 for detailed description of each stage.)

| Experimental group | GSI ($\bar{x}$ ± SEM) | Number of cells/mm$^2$ of gonad ($\bar{x}$ ± SEM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | First growth stage | Perinucleus stage | Yolk vesicle stage | Primary yolk stage | Secondary and tertiary yolk stage | Atretic follicle stage |
| 16L:8D/21°C | 10.5±0.5 ($n_1$=35) | - ($n_2$=19) | 4.92±0.85 | 1.55±0.38 | 3.23±0.4 | 2.38±0.17 | + |
| 8L:16D/21°C | 9.2±0.7 ($n_1$=34) | + ($n_2$=19) | 9.15±1.76 | 2.21±0.43 | 4.16±0.6 | 1.64±0.28 | ++ |
| 16L:8D/12°C | 11.8±0.8 ($n_1$=32) | + ($n_2$=9) | 8.05±2.5 | 0.88±0.2 | 2.83±0.6 | 2.11±0.17 | |
| 8L:16D/12°C | 12.3±0.8 ($n_1$=36) | + ($n_2$=10) | 6.95±2.38 | 1.5 ±0.46 | 3.1 ±0.6 | 1.95±0.37 | |

$n_1$ = total number of fish in the group.

$n_2$ = number of ovaries examined in the group.

Fig. 13. Cross-section through the gonad of a mature female fish. (X30)

f = oogonia and oocytes in the first growth stage

p = oocyte in the perinucleolus stage

py = oocyte in the 1°yolk stage

sy = oocyte in the 3°yolk stage

v = oocyte in the yolk vesicle stage

yg = yolk globules

yv = yolk vesicles

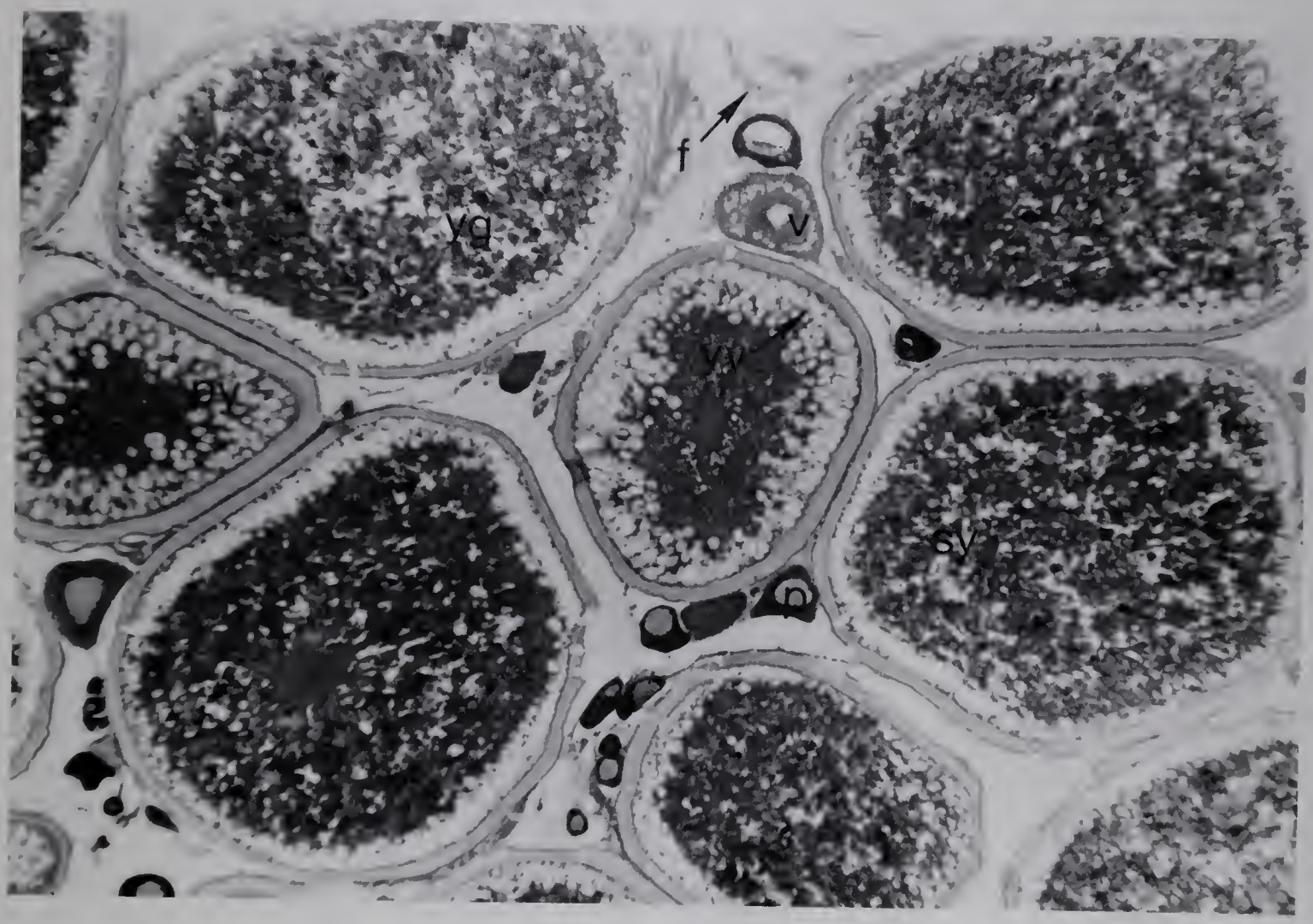

f
yg
v
vv
syl
sy
p

Fig. 14. Variations of serum GTH in a 24 hour period of fish with immature gonads in a quiescent state (regressed fish), females undergoing ovarian recrudescence (maturing females) and females with a mature ovary (mature females), subjected to the 16L:8D/21±1°C regime. Numbers beside each point indicate number of fish sampled at each time. Number of fish in each group and their GSI (mean ± SEM) are indicated on top of the figure.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | | | |
|---|---|---|---|
| 0400 hr | Regressed | Mature | Maturing |
| 0800 hr | Regressed | Mature | Maturing |
| 1200 hr | Regressed | Mature | Maturing |
| 1600 hr | Regressed | Mature | Maturing |
| 2000 hr | Regressed | Mature | Maturing |
| 2400 hr | Regressed | Mature | Maturing |

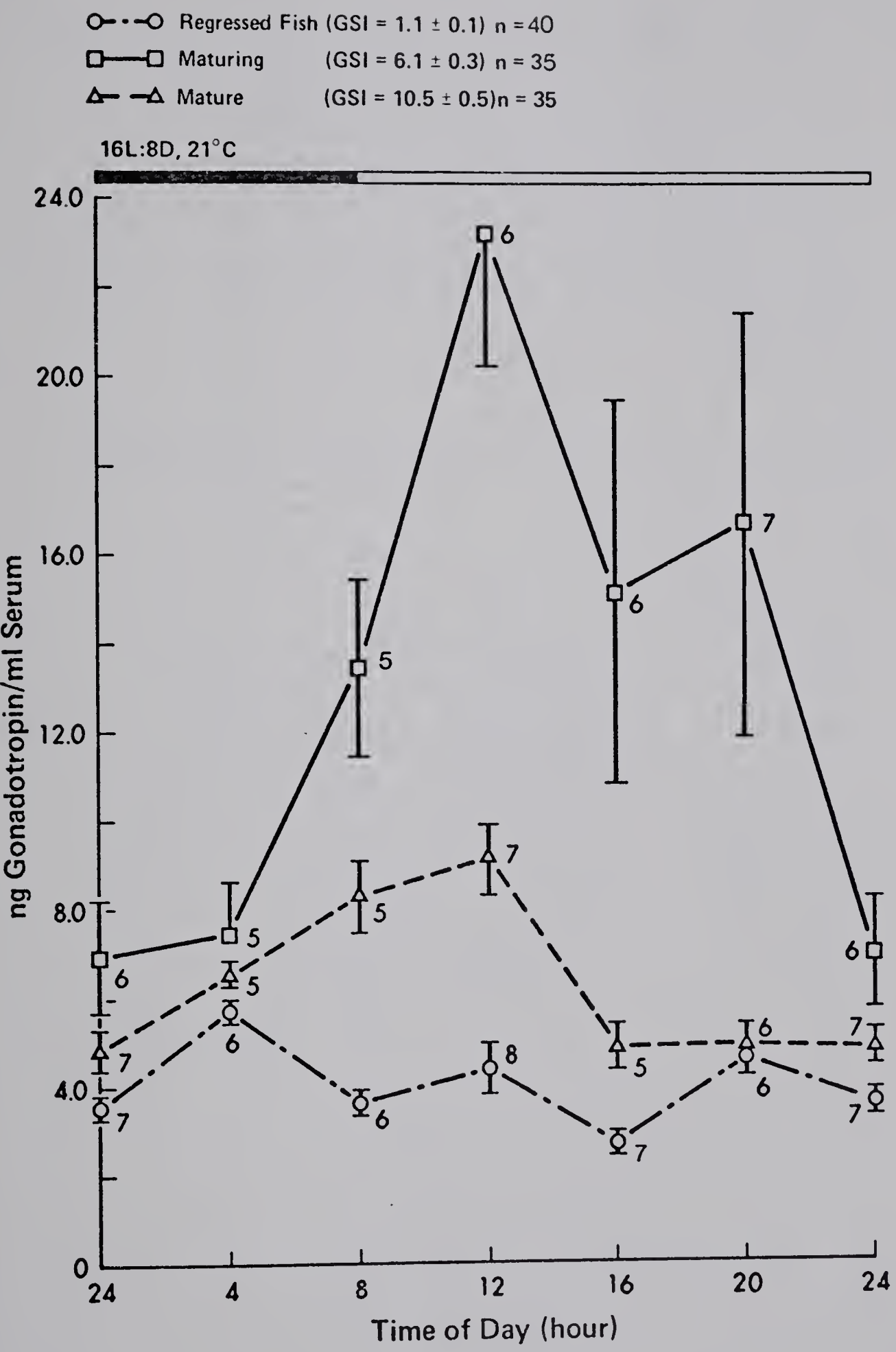

Regressed Fish (GSI = 1.1 ± 0.1) n = 40
Maturing (GSI = 6.1 ± 0.3) n = 35
Mature (GSI = 10.5 ± 0.5) n = 35
16L:8D, 21°C
24.0
20.0
16.0
12.0
8.0
4.0
0
ng Gonadotropin/ml Serum
24
4
8
12
16
20
24
Time of Day (hour)

Fig. 15. Variations of serum GTH in a 24 hour period of regressed fish, maturing females and mature females, subjected to the 8L:16D/21±1°C regime. Numbers beside each point indicate number of fish sampled at each time. Number of fish in each group and their GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:
$p < 0.05$

| | | | |
|---|---|---|---|
| 0400 hr | Regressed | Mature | Maturing |
| 0800 hr | Regressed | Mature | Maturing |
| 1200 hr | Regressed | Maturing | Mature |
| 1600 hr | Regressed | Maturing | Mature |
| 2000 hr | Mature | Regressed | Maturing |
| 2400 hr | Regressed | Mature | Maturing |

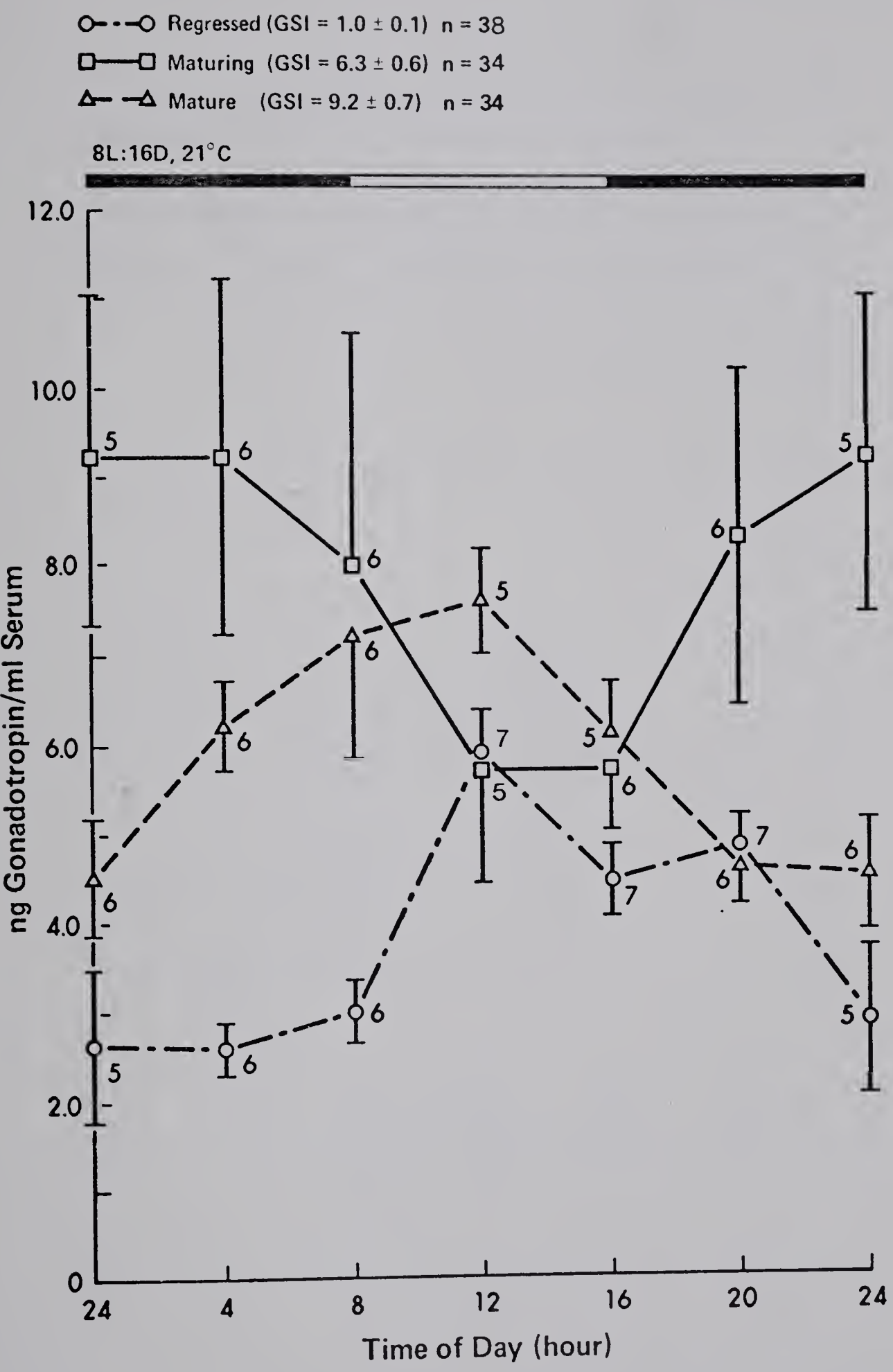

Regressed (GSI = 1.0 ± 0.1) n = 38
Maturing (GSI = 6.3 ± 0.6) n = 34
Mature (GSI = 9.2 ± 0.7) n = 34
8L:16D, 21°C
12.0
10.0
8.0
6.0
4.0
2.0
0
ng Gonadotropin/ml Serum
24
4
8
12
16
20
24
Time of Day (hour)

Fig. 16. Variations of serum GTH in a 24 hour period of regressed fish, maturing females and mature females, subjected to the 16L:8D/12±1°C regime. Numbers beside each point indicate number of fish sampled at each time. Number of fish in each experimental group and their GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | | | |
|---|---|---|---|
| 0400 hr | Regressed | Mature | Maturing |
| 0800 hr | Regressed | Mature | Maturing |
| 1200 hr | Regressed | Mature | Maturing |
| 1600 hr | Regressed | Mature | Maturing |
| 2000 hr | Regressed | Maturing | Mature |
| 2400 hr | Regressed | Maturing | Mature |

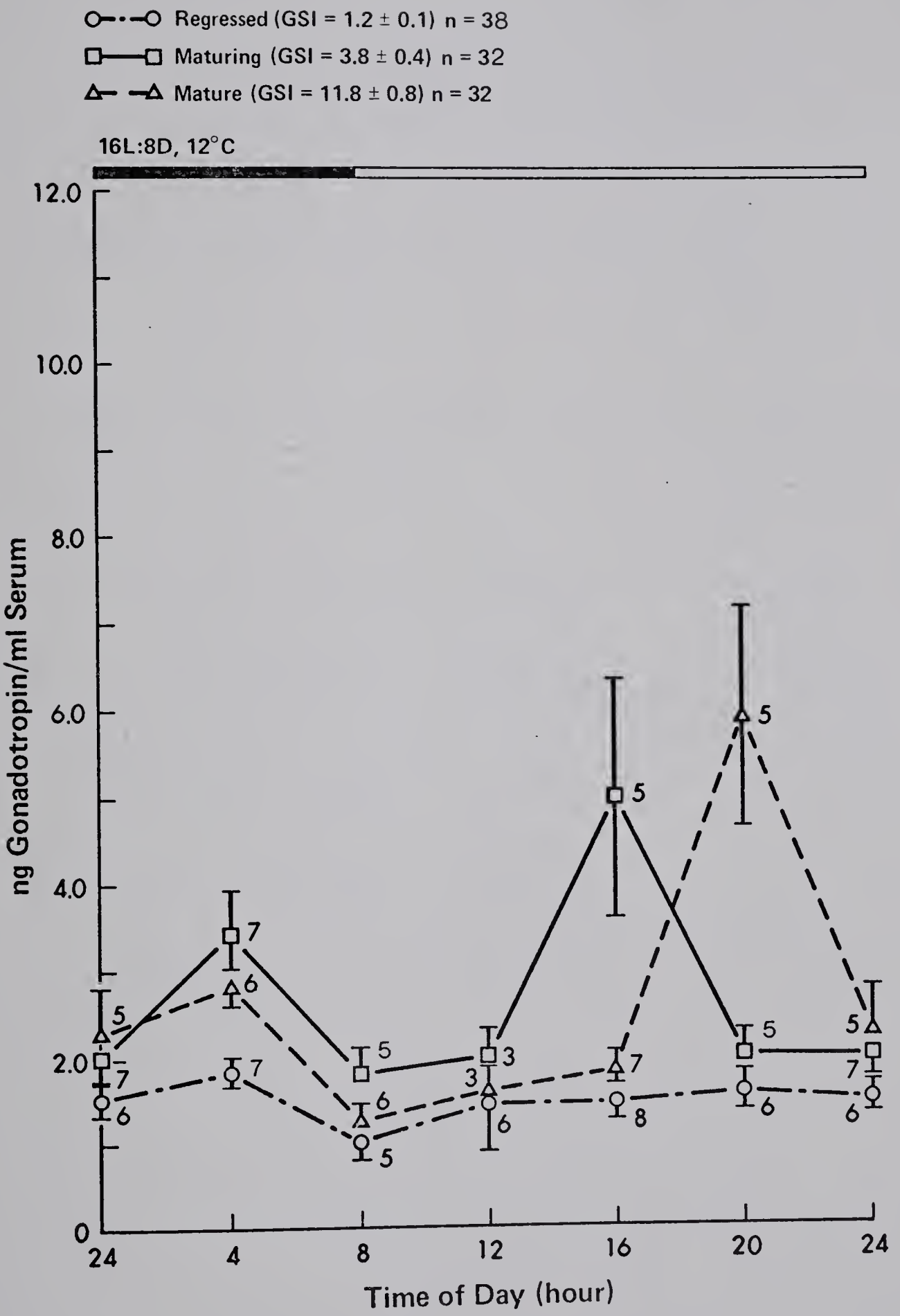
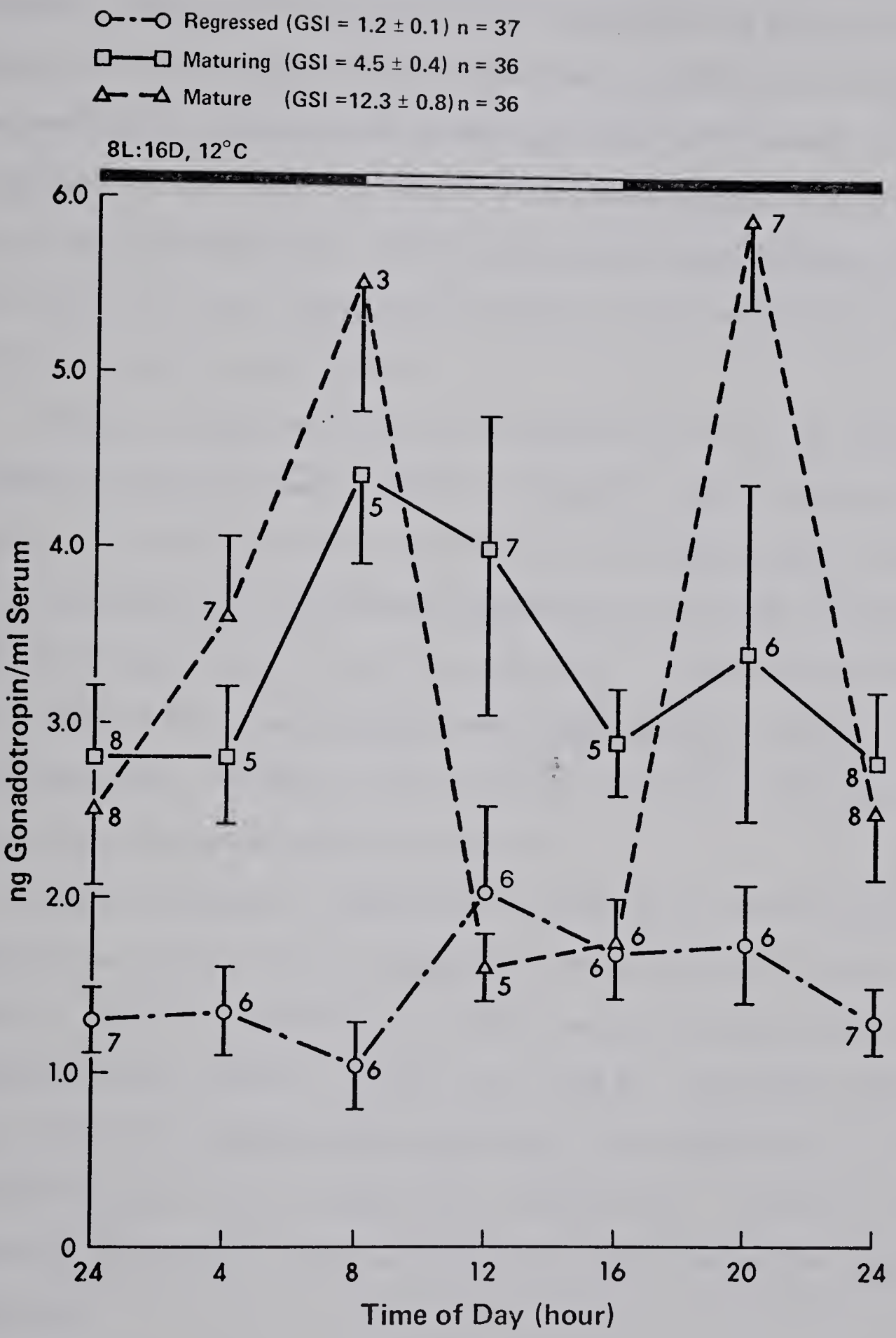

Regressed (GSI = 1.2 ± 0.1) n = 38
Maturing (GSI = 3.8 ± 0.4) n = 32
Mature (GSI = 11.8 ± 0.8) n = 32
16L:8D, 12°C
12.0
10.0
8.0
6.0
4.0
2.0
0
ng Gonadotropin/ml Serum
24
4
8
12
16
20
24
Time of Day (hour)

Fig. 17. Variations in serum GTH over a 24 hour period of regressed fish, maturing females and mature females, subjected to the 8L:16D/12±1°C regime. Numbers beside each point indicate number of fish sampled at each time. Number of fish in each experimental group and their GSI (mean ± SEM) are also indicated.

Computerized results of Duncan multiple range test:

$p < 0.05$

| | | | |
|---|---|---|---|
| 0400 hr | Regressed | Maturing | Mature |
| 0800 hr | Regressed | Maturing | Mature |
| 1200 hr | Mature | Regressed | Maturing |
| 1600 hr | Regressed | Mature | Maturing |
| 2000 hr | Regressed | Maturing | Mature |
| 2400 hr | Regressed | Mature | Maturing |


Regressed (GSI = 1.2 ± 0.1) n = 37
Maturing (GSI = 4.5 ± 0.4) n = 36
Mature (GSI =12.3 ± 0.8) n = 36
8L:16D, 12°C
ng Gonadotropin/ml Serum
6.0
5.0
4.0
3.0
2.0
1.0
0
24
4
8
12
16
20
24
Time of Day (hour)

quiescent state (regressed fish), females undergoing ovarian recrudescence (maturing females) and females with a mature ovary (mature females), subjected to one particular temperature and photoperiod regime. Results from these groups of fish have been described and statistically analysed above; therefore the following section will present only the comparison of the serum GTH levels of the three groups of fish at each individual sampling time. The Duncan multiple range test was used for the analysis of data, computerized results of the tests are presented in the caption for each figure.

Figure 14 shows variations of serum GTH of fish at the three different stages of gonadal maturity, exposed to the 16L:8D/21±1°C regime. At 1200 hr, 1600 hr and 2000 hr, the average value of the maturing groups was significantly higher than the values from the mature females group and the regressed group. At 0800 hr and 2400 hr, the values of the maturing groups were higher than the values of the regressed fish. At 0400 hr, there were no significant differences between the values of the three groups.

Figure 15 shows the variations of serum GTH of regressed fish, maturing and mature females, exposed to the 8L:16D/21±1°C regime. At 0800 hr, 1200 hr and 1600 hr, no significant differences between the three means were found. At 2000 hr and 2400 hr, the average values of serum GTH of the maturing fish were found to be higher than the values of the regressed and the mature fish, respectively. At 0400 hr, the value of the maturing group was higher than the value of the regressed fish only.

Variations of serum GTH of fish at the three different stages of gonadal maturity, subjected to the 16L:8D/12±1°C regime, are shown in

Figure 16. At 1600 hr, the average value of serum GTH of the maturing fish was found to be significantly higher than the values of the mature and regressed fish. At 2000 hr, the value of the mature fish was higher than the value of the regressed fish. At 0400 hr, 0800 hr, 1200 hr and 2400 hr, there were no significant differences between the three means.

Figure 17 shows variations of serum GTH of fish subjected to the 8L:16D/12±1°C regime. At 1600 hr, the value of serum GTH of the maturing fish was found to be higher than the value of the regressed fish. At 0400 hr, 0800 hr and 2000 hr, the values of the mature fish were higher than the values of the regressed fish. At 1200 hr and 2400 hr, no significant differences between the three means were found.

The average GSI of the regressed fish, maturing females and mature females, subjected to a particular photoperiod and temperature regime, were compared using the Student's *t*-test for unpaired values. Under all experimental regimes, the GSI of the mature fish was higher than the GSI of the maturing fish, which was in turn higher than the GSI of the regressed fish.

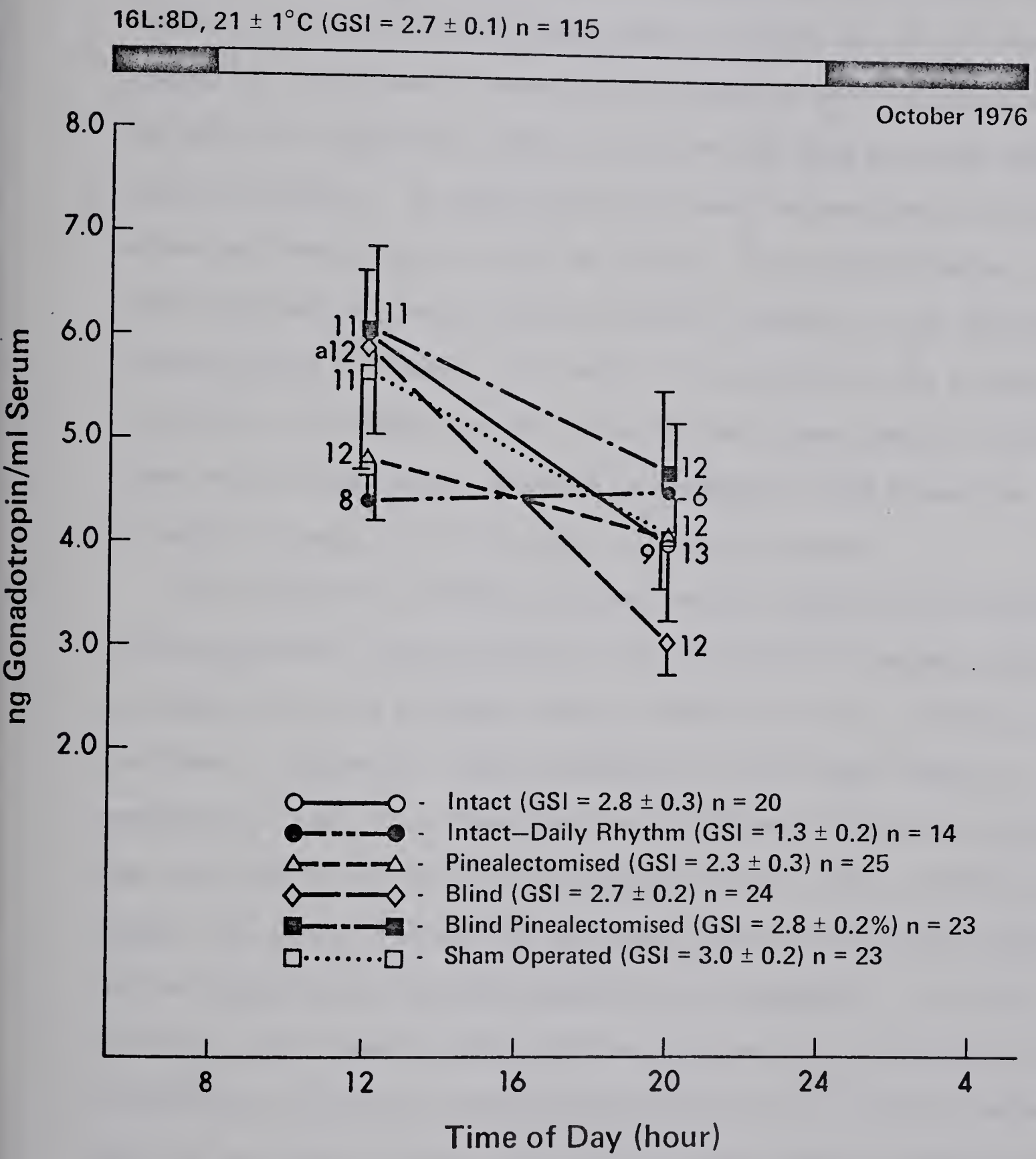
## II. Pinealectomy-Blinding Experiment

*Fish with a regressed gonad*

The variations in serum GTH levels in blinded, pinealectomised, blinded and pinealectomised, sham operated and intact fish with immature gonads in a quiescent state (October 1976), subjected to the 16L:8D/21±1°C regime, are shown in Figure 18. The values of serum GTH at 1200 hr and 2000 hr on the three sampling days (see Fig. 2) were recombined to constitute an average value at 1200 hr and 2000 hr, respectively. All the means were statistically analysed with the

Fig. 18. Serum GTH levels (mean ± SEM) of pinealectomised, blinded, blinded and pinealectomised, sham operated and intact regressed fish, subjected to the 16L:8D/21±1°C regime. The GTH levels of intact regressed fish, exposed to 16L:8D/21±1°C in the daily rhythm experiment are also included. Numbers beside each point indicate number of fish sampled at each time. Number of fish in each experimental group and their GSI (mean ± SEM) are also indicated.

a - significantly higher ($p < 0.05$) than the value of the blinded group at 2000 hr


16L:8D, 21 ± 1°C (GSI = 2.7 ± 0.1) n = 115
October 1976
ng Gonadotropin/ml Serum
8.0
7.0
6.0
5.0
4.0
3.0
2.0
11
11
a12
11
12
8
12
6
12
9
13
12
- Intact (GSI = 2.8 ± 0.3) n = 20
- Intact—Daily Rhythm (GSI = 1.3 ± 0.2) n = 14
- Pinealectomised (GSI = 2.3 ± 0.3) n = 25
- Blind (GSI = 2.7 ± 0.2) n = 24
- Blind Pinealectomised (GSI = 2.8 ± 0.2%) n = 23
- Sham Operated (GSI = 3.0 ± 0.2) n = 23
8
12
16
20
24
4
Time of Day (hour)

Student's *t*-test for unpaired values.

No significant differences were found between the serum GTH levels of the five experimental groups either at 1200 hr or at 2000 hr. The values of serum GTH of the intact group at 1200 hr and 2000 hr were compared to the values of serum GTH of regressed fish subjected to the 16L:8D/21±1°C regime and sampled at 1200 hr and 2000 hr in the daily rhythm experiment. No differences were found between the two 1200 hr values and between the two 2000 hr values. The serum GTH value of each experimental group at 1200 hr was also compared to the value for the same group at 2000 hr. The value of the blinded group at 1200 hr was found to be higher than the value of the blinded group at 2000 hr. There were no significant differences between the 1200 hr and the 2000 hr values in any of the other four experimental groups.

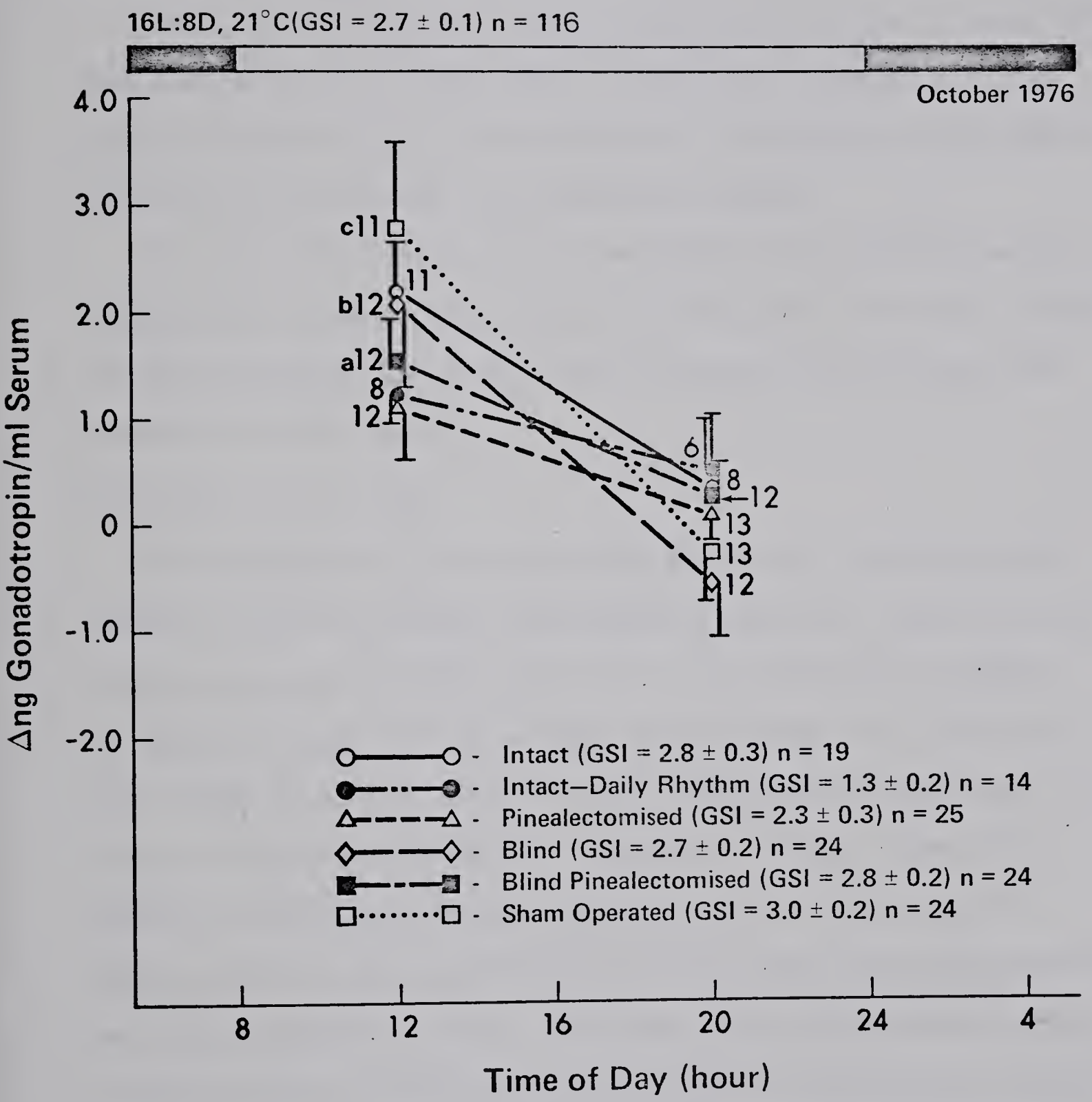
The variations of serum GTH levels of the regressed fish from the five experimental groups exposed to the 16L:8D/21±1°C regime, expressed as changes from the presample taken at 2000 hr on day 4 (see Fig. 2), are shown in Figure 19. (The calculation of the change value is explained on page 31). There were no significant differences between the serum GTH levels of the five groups at either 1200 hr or 2000 hr. Again, the values obtained for the intact group at 1200 hr and 2000 hr in the pinealectomy-blinding experiment were compared to the corresponding values from the daily rhythm experiment and no statistically significant differences were detected between the two 1200 hr values and the two 2000 hr values. The values of each group at 1200 hr were compared to the values for the same group at 2000 hr. The values of the pinealectomised and blinded group, blinded, and sham operated groups

Fig. 19. Serum GTH levels (mean ± SEM) expressed as changes from presample of pinealectomised, blinded, blinded and pinealectomised, sham operated and intact regressed fish, exposed to the 16L:8D/21±1°C regime. GTH levels of intact regressed fish, exposed to 16L:8D/21±1°C in the daily rhythm experiment, are also included. Numbers beside each point indicate the number of fish sampled at each time. Number of fish in each group and their GSI (mean ± SEM) are also indicated.

a - significantly higher ($p < 0.05$) than blind and pinealectomised group at 2000 hr

b - significantly higher ($p < 0.05$) than blind group at 2000 hr

c - significantly higher ($p < 0.05$) than sham operated group at 2000 hr


16L:8D, 21°C(GSI = 2.7 ± 0.1) n = 116
October 1976
4.0
3.0
2.0
1.0
0
-1.0
-2.0
Δng Gonadotropin/ml Serum
c11
11
b12
a12
8
12
6
8
12
13
13
12
Intact (GSI = 2.8 ± 0.3) n = 19
Intact—Daily Rhythm (GSI = 1.3 ± 0.2) n = 14
Pinealectomised (GSI = 2.3 ± 0.3) n = 25
Blind (GSI = 2.7 ± 0.2) n = 24
Blind Pinealectomised (GSI = 2.8 ± 0.2) n = 24
Sham Operated (GSI = 3.0 ± 0.2) n = 24
8
12
16
20
24
4
Time of Day (hour)

at 1200 hr were higher than the values of these same groups at 2000 hr. The 1200 hr and 2000 hr values of the pinealectomised, and intact groups were not significantly different.

Using a paired Student's *t*-test, the absolute values of serum GTH from fish sampled the second time at 2000 hr were compared with the presample values on day 4 of the same fish. There were no significant differences in any of the five experimental groups.

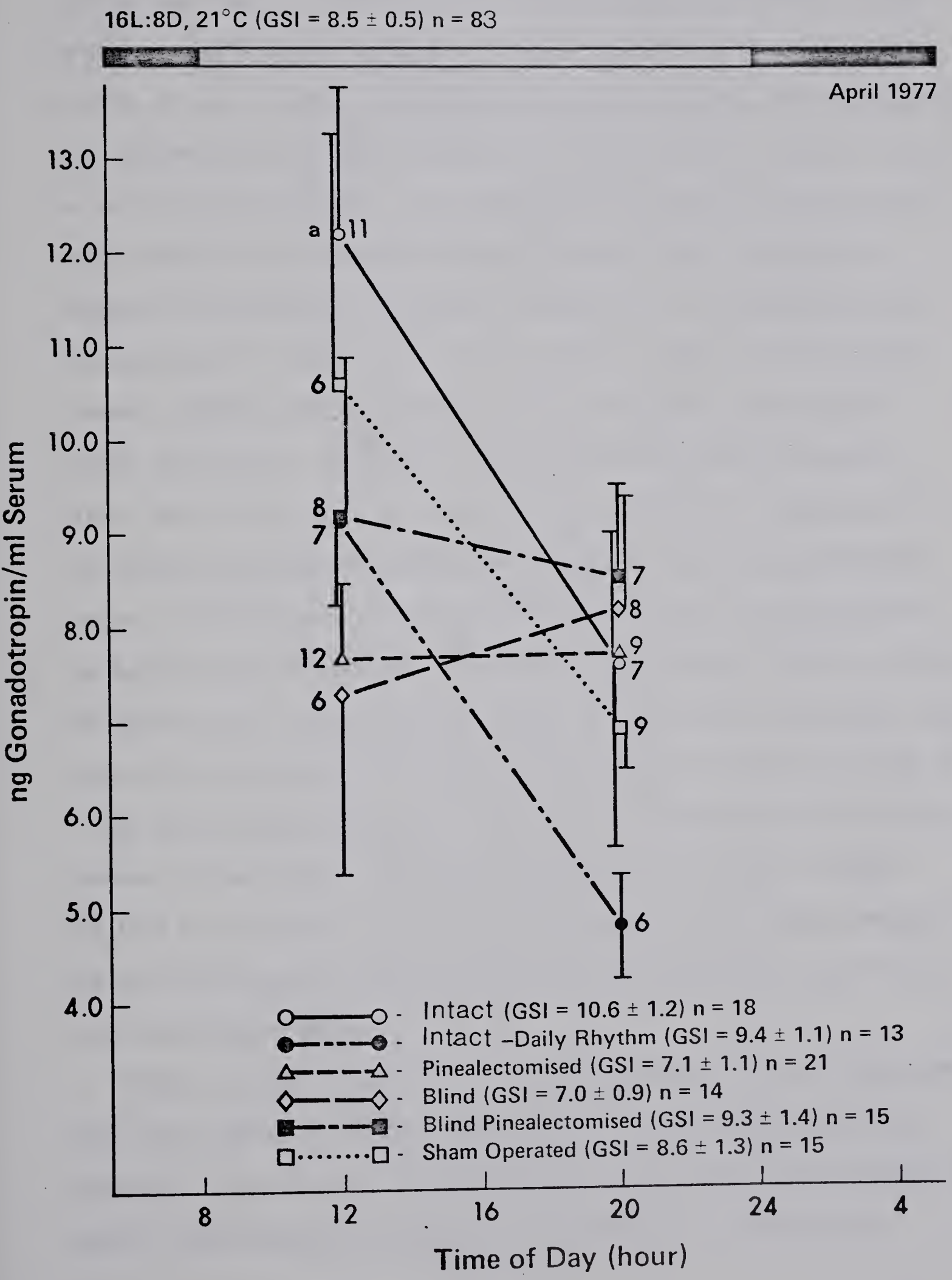
The GSI of the intact group of regressed fish (2.5±0.2%) was not significantly different from the GSI of the blinded (2.7±0.2%), blinded and pinealectomised (2.7±0.2%), pinealectomised (2.3±0.2%) and sham operated (3.0±0.2%) groups.

*Fish with a mature ovary*

The variations of serum GTH levels in blinded, pinealectomised, blinded and pinealectomised, sham operated, and intact female fish with a mature ovary (April 1977), subjected to the 16L:8D/21±1°C regime, are shown in Figure 20. The average value of serum GTH of the intact fish sampled at 1200 hr was found to be significantly higher than the value of the pinealectomised fish sampled at 1200 hr. There were no significant differences between the values of the intact group at 1200 hr and the values of the sham operated, blinded and pinealectomised, and blinded groups at 1200 hr. The values of the sham operated group at 1200 hr were not different from the values of the other four experimental groups at 1200 hr. No significant differences were detected between the values of the five experimental groups at 2000 hr. The values of serum GTH of the intact group at 1200 hr and 2000 hr were compared to the values of the mature female fish subjected to the

Fig. 20. Serum GTH levels (mean ± SEM) of pinealectomised, blinded, blinded and pinealectomised, sham operated and intact fish with a mature ovary, subjected to the 16L:8D/21±1°C regime. GTH levels of intact mature fish exposed to 16L:8D/21±1°C in the daily rhythm experiment are also included. Numbers beside each point indicate number of fish sampled at each time. Number of fish in each experimental group and their GSI (mean ± SEM) are also indicated.

a - significantly higher ($p < 0.05$) than value of pinealectomised group at 1200 hr

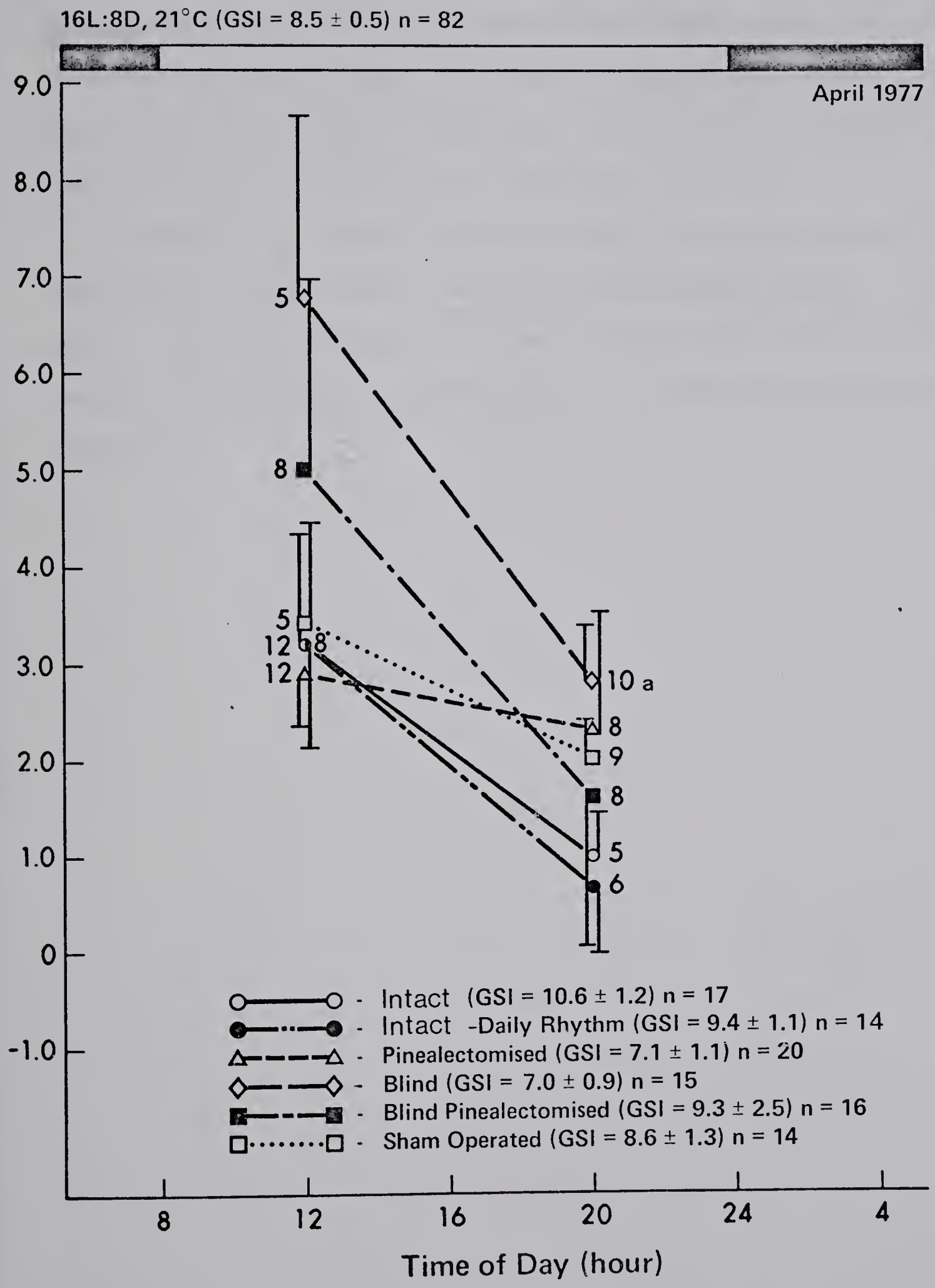

16L:8D, 21°C (GSI = 8.5 ± 0.5) n = 83
April 1977
ng Gonadotropin/ml Serum
13.0
12.0
11.0
10.0
9.0
8.0
6.0
5.0
4.0
a
11
6
8
7
12
6
7
8
9
7
9
6
Intact (GSI = 10.6 ± 1.2) n = 18
Intact –Daily Rhythm (GSI = 9.4 ± 1.1) n = 13
Pinealectomised (GSI = 7.1 ± 1.1) n = 21
Blind (GSI = 7.0 ± 0.9) n = 14
Blind Pinealectomised (GSI = 9.3 ± 1.4) n = 15
Sham Operated (GSI = 8.6 ± 1.3) n = 15
8
12
16
20
24
4
Time of Day (hour)

16L:8D/21±1°C regime and sampled at 1200 hr and 2000 hr in the daily rhythm experiment. The two 1200 hr values and the two 2000 hr values were not significantly different. Also, the 1200 hr values and the 2000 hr values of each experimental group were compared and no significant differences were found between the 1200 hr and the 2000 hr values in any of the five groups. The variations of serum GTH levels of the mature female fish from the five experimental groups, expressed as changes from presample, are shown in Figure 21. The value of serum GTH expressed as change in the intact fish at 1200 hr was not significantly different from the values of the other four experimental groups at 1200 hr. At 2000 hr, the value of the blinded group was higher than the value of the intact group at 2000 hr. The values of the blinded and pinealectomised, sham operated, and pinealectomised groups at 2000 hr were not significantly different from the value of the intact group at 2000 hr. The values of the intact fish at 1200 hr and 2000 hr were compared to the values of serum GTH of the mature fish exposed to the 16L:8D/21±1°C regime and sampled at 1200 hr and 2000 hr in the daily rhythm experiment. There were no significant differences between the two 1200 hr values and between the two 2000 hr values. The 1200 hr and 2000 hr values of each particular group were compared and no statistically significant differences were found in any of the five experimental groups.

Using a paired Student's *t*-test, the absolute values of serum GTH from fish sampled the second time at 2000 hr were compared with the presample values on day 4 of the same fish. The mean 2000 hr values of intact, sham operated and pinealectomised fish were found to be

Fig. 21. Serum GTH levels (mean ± SEM) expressed as changes from the presample of pinealectomised, blinded, pinealectomised and blinded, sham operated and intact fish with a mature ovary, subjected to the 16L:8D/21±1°C regime. GTH levels of intact mature fish, exposed to 16L:8D/21±1°C in the daily rhythm experiment, are also included. Numbers beside each point indicate number of fish sampled at each time. Number of fish in each experimental group and their GSI (mean ± SEM) are also indicated.

a - significantly ($p < 0.05$) higher than the intact group at 2000 hr


16L:8D, 21°C (GSI = 8.5 ± 0.5) n = 82
April 1977
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
-1.0
Δng Gonadotropin/ml Serum
5
8
5
12
8
12
10 a
8
9
8
5
6
- Intact (GSI = 10.6 ± 1.2) n = 17
- Intact -Daily Rhythm (GSI = 9.4 ± 1.1) n = 14
- Pinealectomised (GSI = 7.1 ± 1.1) n = 20
- Blind (GSI = 7.0 ± 0.9) n = 15
- Blind Pinealectomised (GSI = 9.3 ± 2.5) n = 16
- Sham Operated (GSI = 8.6 ± 1.3) n = 14
8
12
16
20
24
4
Time of Day (hour)

significantly higher than their presample values. The 2000 hr values of the blinded and pinealectomised fish were not significantly different from their presample values. The 2000 hr values of the blinded fish were not different from their presample values; however, the value of the calculated $t$ was close to the significant value of $t$.

The GSI of the intact females was found to be significantly higher than the GSI of the blinded, and of the pinealectomised groups. There were no significant differences between the GSI of the intact group and the GSI of the blinded and pinealectomised, or the sham operated group, respectively.

# DISCUSSION

## I. Daily Rhythms in Serum GTH Levels

Investigations reported in this study present evidence for the existence of daily variations in serum GTH levels in the goldfish *Carassius auratus*. The pattern of these variations in serum GTH in a 24 hour period varies depending on the stage of gonadal maturity of the fish and the conditions of photoperiod and temperature to which the fish are exposed.

The results in the daily rhythm experiments have been expressed in two ways: as absolute values and as differences from the presample. The absolute values have been provided by samples taken on days 7, 8 and 9, and reconstructed in such a way as to constitute a 24 hour period. Each sample is provided by a group of fish which were sacrificed after sampling, since goldfish of the size used cannot reform blood at a sufficient rate for subsequent sampling (personal observation), and also because repeated sampling will probably stress the fish. The sampling was spread over three days in order to allow the animals remaining in the aquarium after each sample was taken to have time to recover from the possible stress caused by the opening of the tank and the netting of some fish from the tank. A time period of 8 or 12 hours between each subsequent sampling was considered sufficient for such recovery. The basic assumption of this approach is that the GTH levels in the 24 hour period of days 7, 8 and 9 are similar. The use of the presample values at 2000 hr on day 4 and the comparison of these values to the 2000 hr values on day 7, was expected

to provide some information about the validity of this assumption. If the 2000 hr values of the presample on day 4 and the 2000 hr values on day 7 are not significantly different, it is assumed that the patterns of daily variations in GTH levels occurring on days 4 and 7 (and presumably days 8 and 9) are all similar. If the 2000 hr values on day 4 and day 7 are different, it probably means that the pattern of daily fluctuations in serum GTH levels are different or changing under the conditions used. Results presented in this study show that the approach using samples from three consecutive days was justified since the presample values at 2000 hr on day 4 were not significantly different from the 2000 hr values on day 7 in 11 of the 12 experimental groups.

The experimental design using the presample values was also expected to minimize individual variability between the fish. By comparison of the second sample to the presample value, each fish has a point of reference which can be used as a control for differences in the serum GTH levels between the fish. Since in all the daily rhythm experiments the pattern of daily variations in GTH levels in fish subjected to one particular set of environmental conditions were fairly similar, whether the GTH levels were expressed as absolute values or changes from the presample, it seems that variability in the responses by the fish to each set of environmental conditions was small. The time of the presample therefore functions as an arbitrary point of reference for each fish. The specific time (2000 hr) was chosen by referring to the study by Breton *et al.* (1972) who determined that the lowest plasma GTH levels were detected at 2000 hr in mature female

goldfish exposed to outdoor summer conditions of photoperiod and temperature. Therefore 2000 hr was chosen as the presample time, expecting that all the changes in GTH levels during the 24 hr period would be positive values, at least in mature females subjected to the 16L:8D/21±1°C regime.

*Regressed fish*

The daily rhythm experiment on fish having immature gonads in a quiescent state (also designated as regressed fish in this study) provided evidence that the fish in this particular state of gonadal maturity have either small or no diurnal variations in serum GTH levels under different photoperiod and temperature conditions.

No significant variations in serum GTH, when expressed either as absolute values or as differences from the presample, occurred in a 24 hour period in the two groups exposed to cold temperature (16L:8D/12±1°C, 8L:16D/12±1°C). The two groups subjected to warm temperature, and long or short photoperiod (16L:8D/21±1°C, 8L:16D/21±1°C), showed significant peaks in serum GTH in a 24 hour period when the GTH levels were expressed as absolute values (without consideration of the presample). However, only the peak in the 8L:16D/21±1°C group was significant when the GTH levels were expressed as changes from the presample. Thus, there is no clear evidence for a rhythm under the 16L:8D/21±1°C regime. The fact that some small fluctuations in GTH levels occurred in fish exposed to the warm temperature, and also the fact that at a number of sampling times in the 24 hour period the GTH levels of the warm fish were higher than the GTH levels of the cold fish, indicates a stimulatory effect of warm temperature on GTH

secretion. However, when both the absolute values and the changes from the presample are considered, it is apparent that in general, the GTH levels in all groups of regressed fish are fairly similar and rather uniform throughout the 24 hour period.

Evidence in the literature suggests that some of the experimental regimes used in this study could be stimulatory to GTH secretion in the regressed goldfish. Ahsan (1966a) reported that low temperature (9±1.5°C) stimulated testicular growth whereas high temperature (19.5±1.5°C) suppressed testicular growth in the lake chub, *Couesius plumbeus* in the postspawning phase of the reproductive cycle. He also found that a short photoperiod (8L:16D) stimulated the maturation process in the postspawning fish. On the other hand, de Vlaming (1975) showed that long photoperiod and warm temperature (15.5L:8.5D/25°C) stimulated gonadal development in the golden shiner, *Notemigonus crysoleucas* in the postspawning period. Long photoperiod and warm temperature have also been reported to accelerate ovarian recrudescence in the green sunfish, *Lepomis cyanellus* (Kaya and Hassler, 1972), shiner sea perch, *Cymatogaster aggregata* (Wiebe, 1968a) and the Indian catfish, *Heteropneustes fossilis* (Anand *et al.*, 1974; Viswanathan *et al.*, 1974), which belongs to the same order (*Cypriniformes*) as the goldfish. Baggerman (1972) demonstrated that although long photoperiod and warm temperature accelerate gonadal recrudescence of the threespine stickleback, *Gasterosteus aculeatus* in spring and winter, these conditions do not stimulate gonadal development in the summer. This suggests that *G. aculeatus* is refractory to long photoperiod and warm temperature in the postspawning period. These studies used either

the GSI or histological indices to demonstrate the effect of the environment on the gonadal development. Gillet *et al.* (1976) measured the GTH levels and determined the GTH content in the plasma of male goldfish exposed to different temperature regimes. He demonstrated the stimulatory effect of warm temperature on GTH secretion in maturing males in February by showing that GTH levels of fish exposed to temperatures above 17°C were higher than GTH levels of fish at 10°C. Although sexually regressed fish were not included in the study by Gillet *et al.* (1976), the fact that warm temperature stimulated GTH secretion in maturing animals could possible imply that warm temperature could also stimulate GTH secretion in regressed fish. The detected levels of GTH, and either small peaks or absence of peaks in serum GTH in a 24 hour period in all groups of sexually regressed fish in the present study, suggest that the hypothalamo-pituitary axis of fish with immature gonads in a quiescent state is relatively unresponsive, or refractory, to a short term exposure to conditions which might be considered as stimulatory for GTH secretion. It should be noted also that the GSIs of the four experimental groups of regressed fish, exposed to different conditions of photoperiod and temperature, were almost identical and histological examination of their gonads showed that they were all in a similar sexually immature state, regardless of the experimental photoperiod and temperature regime.

In general, the reproductive systems of some animals become refractory in order for the organism to recover from the physiologically exhaustive process of breeding, and to assure the production of young at the proper time in the next breeding season. Little is known

about the mechanisms controlling this in fishes. Weil *et al.* (1975) demonstrated that the stimulation of GTH secretion following administration of exogenous LH-FSH/RH into sexually regressed carp, *Cyprinus carpio*, is less pronounced than the stimulation of GTH secretion in maturing or mature animals. Billard *et al.* (1976) showed that the increase in GTH secretion following castration is smaller in rainbow trout, *Salmo gairdneri*, having quiescent gonads than in rainbow trout undergoing ovarian recrudescence or mature trout. These studies suggest that the hypothalamus and the pituitary gland are important factors involved in the control of the onset and the termination of the refractory period in fishes.

Data provided in this study demonstrated that the pattern of serum GTH levels in a 24 hour period in fish having immature gonads in a quiescent state (regressed fish) is characterized by relatively low GTH levels and a general absence of fluctuations in these levels. The fact that this also occurs in regressed fish exposed to environmental conditions, which may be considered as stimulatory for the GTH secretion, seems to indicate that the regressed fish are refractory to a short term exposure to these environmental conditions, and that the refractoriness of the neuroendocrine system involved in GTH secretion is reflected by an absence of daily fluctuations in serum GTH levels.

*Females undergoing ovarian recrudescence*

Maturing female fish show daily fluctuations in serum GTH levels in the 24 hour period and the pattern of these fluctuations varies depending on the experimental photoperiod and temperature regime.

Fish subjected to the 16L:8D/21±1°C regime show marked fluctuations in serum GTH levels. A large peak of GTH (average = 24 ng/ml of serum) was detected around 1200 hr (4 hours after the onset of light) and relatively low GTH levels (about 7 ng/ml of serum) were detected at 2400 hr and 0400 hr. Warm temperature alone does not seem to generate pronounced daily fluctuations in the serum GTH levels in the maturing females since significant daily variations were not detected in the 8L:16D/21±1°C, and the large peak of serum GTH around 1200 hr found in the long photoperiod group was also absent. Daily variations in serum GTH levels occurred in the maturing fish exposed to the cold temperature, the general pattern being the presence of either one or two peaks. When absolute values for the 16L:8D/12±1°C group are considered, one peak occurring at 1600 hr is apparent. On the other hand, consideration of GTH values expressed as change from the presample indicates the presence of two peaks, one at 0400 hr and the second one at 1600 hr. In the 8L:16D/12±1°C group, no significant peaks were detected when absolute values of GTH were used, but the serum GTH levels between 0800 hr and 2000 hr were found to be higher than the other values in the 24 hour period when GTH levels were expressed as change from the presample. This indicates that in fish exposed to cold temperature, peaks in serum GTH tend to occur either around the onset of light and/or 8-12 hours after the onset of light. When the maturing fish are compared with the mature fish in the general discussion, more support for this hypothesis will be provided.

The 16L:8D/21±1°C regime seems to strongly stimulate GTH secretion in the maturing females. Fish exposed to this regime showed a

large peak in serum GTH four hours after the onset of light. It is interesting to note that relatively low GTH levels were also detected at certain times of the 24 hour period in this group, despite the presence of the large peak of serum GTH. Another indication of the stimulatory effect of warm temperature and long photoperiod is the fact that the GTH levels of the 16L:8D/21±1°C group are generally significantly higher than the GTH levels of the two cold groups; at only a few sampling times are both warm groups higher than the two cold groups. It should be noted also that the presample values and the 2000 hr values on day 7 are different in the 16L:8D/21±1°C group, but not in the other three groups. This probably signifies that under the short term exposure to the long photoperiod and warm temperature regime, the pattern of daily fluctuations in serum GTH levels is undergoing some change; possibly the peak at 1200 hr is gradually increasing, indirectly causing the value at 2000 hr to increase also. It seems that this does not occur under short term exposure to short photoperiod and warm temperature or under the relatively longer exposure (see Fig. 1) to cold temperature. The high levels of serum GTH found at 1200 hr in the 16L:8D/21±1°C group may also be interpreted as evidence supporting the hypothesis of the stimulatory effect of warm temperature and long photoperiod on gonadal recrudescence. Warm temperature certainly appears as an important stimulatory factor for the regulation of GTH secretion since even in the 8L:16D/21±1°C group, which did not have significant daily fluctuations in serum GTH, the GTH levels were still relatively high compared to the values of the cold groups.

Evidence from the comparison of GSIs of the four experimental groups provide some further support for the hypothesis of the stimulatory effect of warm temperature. The GSIs of both warm groups were both significantly higher than the GSIs of the cold groups, despite the fact that the experiment using fish exposed to 12°C was done 2 weeks later than the experiment using fish exposed to 21°C and therefore the cold fish had 2 more weeks for development in the main holding tank (see Fig. 1). Histological examination of the gonads provides some further evidence in this regard. Ovaries of fish from the two cold groups seem to contain more yolkless elements (oocytes in the peri-nucleolus stage) than the ovaries from the warm fish. Since a larger amount of yolkless elements in the ovary indicates a less advanced development of the gonad, this suggests that the ovaries of the cold fish were less mature than the ovaries of the warm fish.

The stimulatory effect of long photoperiod and warm temperature on gonadal recrudescence has been demonstrated in the green sunfish, *Lepomis cyanellus* (Kaya and Hassler, 1972), shiner sea perch, *Cymatogaster aggregata* (Wiebe, 1968a), threespine stickleback, *Gasterosteus aculeatus* (Baggerman, 1972), the Indian catfish, *Heteropneustes fossilis* (Viswanathan *et al.*, 1974; Sundararaj and Vasal, 1976) and in the cyprinid, *Notemigonus crysoleucas* (de Vlaming, 1975). Ahsan (1966a) demonstrated the stimulatory effect of short photoperiod and low temperature on testicular development in the cyprinid *Couesius plumbeus* in winter. However, he suggested that the latter stages of spermiogenesis are stimulated as the temperature increases. Kawamura and Otsuka (1950) showed that long photoperiod can stimulate gonadal

maturation in the goldfish *Carassius auratus* in winter if temperature is warm. Also Gillet *et al.* (1976) reported that the plasma GTH levels of male goldfish in February are higher when the fish are exposed to temperatures above 17°C than when they are held at 10°C. These findings are in agreement with data reported in this study. In addition, the present study indicates that the mechanism for the stimulation of gonadal recrudescence under conditions of long photoperiod and warm temperature is by a large surge in secretion of GTH during each 24 hour period, rather than by a continuous high rate of secretion of GTH as suggested by data from Gillet *et al.* (1976).

*Females with a mature ovary*

The serum GTH levels of mature females show fluctuations in a 24 hour period and the pattern of these fluctuations varies depending on the experimental environmental regime imposed.

A peak in serum GTH was detected at 1200 hr (four hours after the onset of light) in the mature females under 16L:8D/21±1°C. A study by Breton *et al.* (1972) provides some support for the existence of this peak and its timing. They measured plasma GTH levels over a 24 hour period in goldfish subjected to outdoor conditions of temperature and photoperiod in July. The temperature varied between 20°C and 30°C during the 24 hour period and it can be assumed that the photoperiod was long, with the light phase starting around 0500 hr. A significant peak in plasma GTH of the mature females was detected at 1100 hr, about 6 hours after the onset of light. Considering the four hours intervals between each sampling in the 16L:8D/21±1°C group, data by Breton *et al.* (1972) on the daily cycle of GTH are similar to the results reported in the present study.

The pattern of GTH fluctuations in the 8L:16D/21±1°C group appears similar to the pattern in the 16L:8D/21±1°C group. However, only when the GTH values are expressed as changes from the presample are the values at 4 hours after the onset of light significantly higher than the other values in the 8L:16D/21±1°C group, In the groups exposed to cold temperature, peaks in serum GTH levels occur at either 12 hours after the onset of light and/or around the onset of light. In the 16L:8D/12±1°C group, a peak in serum GTH was detected at 2000 hr, and in the 8L:16D/12±1°C group, two peaks were detected, at 0800 hr and 2000 hr, respectively. The difference in the number of peaks of serum GTH occurring in the 24 hour period in the two groups exposed to cold temperature could possibly be attributed to the different photoperiod regimes to which the two groups are exposed. The fact that similar patterns of daily fluctuations of serum GTH levels were observed in the groups of maturing females exposed to the cold temperature supports this hypothesis.

Warm temperature seems to stimulate GTH secretion in the mature fish, as it does in maturing fish. Comparison of GTH levels of the four experimental groups at different sampling times shows that either the GTH levels of the warm groups (exposed to long or short photoperiod) are higher than GTH levels of the cold groups, or no differences between the values of the four groups are found. This suggests that the warm temperature alone stimulates GTH secretion in females with a mature ovary, while long photoperiod has no apparent effect. However, the fact that the peak in serum GTH in the 8L:16D/21±1°C group is present only when the GTH values are expressed as changes from the presample could

possibly be interpreted as an indication that the warm temperature alone is slightly less stimulatory to GTH secretion than warm temperature in combination with long photoperiod. De Vlaming (1975) reported that a warm temperature-long photoperiod regime stimulates spawning in the cyprinid *Notemigonus crysoleucas* while long photoperiod or warm temperature alone does not. On the other hand, Ahsan (1966a) showed that final stages of spermiogenesis and spermiation occur at high temperatures in *Couesius plumbeus* in the prespawning condition, photoperiod having only a minor effect. Gillet *et al.* (1976) showed that male goldfish in March have higher plasma GTH levels when they are exposed to 24°C than when they are exposed to 10°C. The effects of photoperiod were not investigated. Data presented here show that in mature females warm temperatures stimulate GTH secretion, while photoperiod has a minor stimulatory effect.

Interesting information is provided by comparison of GSIs of the four experimental groups and histological examination of gonads. The fish exposed to the 8L:16D/12±1°C regime, one which considering the natural environment of the fish might be expected to be the least stimulatory for the final stages of gonadal maturation, had a higher GSI than fish from both warm groups. Histological examination of the gonads provides some insight concerning this. The gonads of fish exposed to the warm temperature contained some atretic follicles while the ovaries of the fish exposed to cold temperature tended not to contain any atretic follicles. It should be noted that the two groups of fish exposed to cold temperature were sampled about 21 days after completion of the experiments with the fish exposed to warm temperature;

therefore the two cold groups had 21 more days for development under the conditions of photoperiod and temperature of the main room tank (see Fig. 1). Ahsan (1966a) reported that the regression of mature testis in *Couesius plumbeus* occurs more rapidly at high temperature than at low temperature. Therefore, in the present study, it seems that under the stimulatory action of the warm temperature, the last stages of gonadal maturation and possibly the very early stages of gonadal regression were initiated, while under the cold temperature regime, gonadal regression did not start. This was reflected by the lower GSI of the fish exposed to warm conditions.

The present data show that mature fish generally have fluctuations in the serum GTH levels in a 24 hour period, and the pattern of these fluctuations varies depending on the conditions of photoperiod and temperature used. Warm temperature seems to stimulate GTH secretion and this is reflected by the occurrence of a peak in serum GTH 4 hours after the onset of light in fish subjected to the 16L:8D/21±1°C and 8L:16D/21±1°C regime. Long photoperiod seems to play a minor stimulatory role. The fact that both groups exposed to warm temperature have similar patterns of serum GTH levels could indicate that the warm temperature and the onset of light are important environmental cues used by the mature fish to regulate the synthesis and/or release of GTH. The similarity in the patterns and levels further suggests that a long photoperiod is not highly stimulatory in mature female goldfish.

*General discussion*

Comparison of the patterns of fluctuations of serum GTH levels in regressed fish, females undergoing ovarian recrudescence and mature

females shows that under the same set of photoperiod and temperature conditions, the regressed fish have fairly uniform low GTH levels in the 24 hour period while the maturing and mature fish have GTH levels that vary during the 24 hour period, the basal level being about that found in the regressed fish. In addition, the patterns of fluctuations found in the maturing and mature fish are frequently similar.

In the 16L:8D/21±1°C groups, the regressed fish have relatively uniform serum GTH levels of about 4 ng/ml of serum while both the mature and maturing females exhibit a peak in serum GTH at 4 hours after the onset of light. The fact that in both these groups the peak occurs at the same time seems to indicate that the onset of light may be a possible cue used by the fish to regulate the GTH secretion under these conditions. The peak in GTH levels is significantly higher in the maturing fish than in the mature fish. Two possible explanations can be provided for this difference. One possibility is that the hypothalamo-pituitary axis of the maturing females, animals which are in the process of gonadal development, might be more responsive to the stimulatory conditions of long photoperiod and warm temperature than the hypothalamo-pituitary axis of the mature females, which have already completed gonadal development. The second explanation involves the negative feedback effect by the sex steroids on the pituitary. The GSI of the maturing females was lower than the GSI of the mature females, and it is possible that the negative feedback effect is stronger in the mature fish than in the maturing fish. Schreck and Hopwood (1974) reported that the estrogen and androgen concentrations in plasma of female goldfish were higher during the spawning season that in winter. Billard *et al.*

(1976) demonstrated that the negative feedback effect is stronger in spermiating male trout, *Salmo gairdneri*, than in males which have not completed all stages of testicular development. Therefore, it may be that the stronger feedback inhibition on the hypothalamo-pituitary axis in the mature fish resulted in a smaller peak of serum GTH in the present study. It should be noted also that the GTH peak values determined in the maturing females are similar to the GTH levels found on the day of ovulation in goldfish by Breton *et al.* (1972). Therefore such a large peak in GTH in the mature fish in this study might stimulate ovulation and the fish are perhaps holding GTH secretion in check, thus preventing ovulation until more appropriate conditions arise.

Data concerning the groups subjected to warm temperature and short photoperiod are difficult to interpret. A peak in serum GTH 4 hours after the onset of light was detected in the regressed fish and possibly also in the mature fish, while there were no significant fluctuations in the GTH levels in the maturing fish. It is difficult to provide an explanation for the fact that the regressed fish show fluctuations in serum GTH levels only under this particular regime while maturing fish do not. It may be that the short photoperiod and warm temperature regime which the goldfish does not experience in its natural environment affects the GTH secretion in an unpredictable way. Fish exposed to cold temperature follow the general trend of responses exhibited by the 16L:8D/21±1°C group. While the regressed fish have uniform low GTH levels, the GTH levels of both maturing and mature fish exhibit similar fluctuations above the basal level, this level being similar to the GTH level determined in the regressed fish. Two significant peaks in

serum GTH tend to occur in the cold groups. The difference in the timing of the peaks between the two cold groups exposed to different photoperiods might be an indication that the length of the light phase and the offset of light could be important cues used by the cold fish.

A characteristic of the patterns of serum GTH fluctuations in regressed, maturing and mature fish, subjected to the same temperature and photoperiod, consistently detected under all environmental regimes, is that during a portion of the 24 hour period, the GTH levels of the three groups are similar. This seems to indicate that the GTH levels of regressed fish represent the basal level while the daily variations of GTH observed in the maturing and mature females represent fluctuations above this basal level (Peter and Hontela, 1977). This is a rather unexpected finding since it was proposed previously, on the basis of evidence from several species of salmonids (Crim *et al.*, 1975; Breton *et al.*, 1975b; Breton *et al.*, 1977) and the tench (Breton *et al.*, 1975b) that serum or plasma GTH levels gradually increase as the gonads progressively mature, from a low GTH level detected in immature fish to higher values in mature fish. Data presented in this study show that under the same environmental conditions the significant difference between fish with immature gonads in a quiescent state, fish undergoing ovarian recrudescence and mature females, is that the latter two groups have greater daily fluctuations above the basal level of serum GTH. Since these two groups are in a more advanced stage of gonadal development than the fish with immature gonads in a quiescent state, this data suggest that the surges of GTH at specific times during the 24 hour period could be physiologically more important than the basal GTH levels.

No information about the mechanism of action of the GTH surges is available yet; however, one possible hypothesis is that GTH acts synergistically with other hormones at certain times of the 24 hour period. Some evidence concerning temporal synergisms of circadian hormone rhythms has been reported in the literature. A daily rhythm of fattening response to prolactin entrained by a daily photoperiod or daily injections of corticosteroids has been demonstrated in several species of teleosts (Lee and Meier, 1967; Meier, 1969; Mehrle and Fleming, 1970; de Vlaming and Sage, 1972; Meier and Burns, 1976). These studies showed that prolactin elicits a change in the fat stores in the body but the extent of this change depends on the time of injection of the prolactin and/or time of injection of corticosteroids. Since circadian variations in circulating prolactin levels have been shown to occur in *Carassius auratus* (Leatherland and McKeown, 1973; McKeown and Peter, 1976; Spieler and Meier, 1976), *Oncorhynchus nerka* (Leatherland *et al.*, 1974), *Fundulus grandis* and *Mugil cephalus* (Spieler, 1975), and circadian variations of corticosteroid levels occur in the *Carassius auratus* (Peter and Hontela, unpublished results), this provides the possibility that circadian prolactin and corticosteroid rhythms may synchronize in some way to act synergistically at specific times of the day. It is also possible that a synergistic action of GTH, prolactin and corticosteroids at certain times of the day may influence the fat metabolism in the gonad and therefore influence gonadal growth. Other hormones which could be expected to act in temporal synergism with the GTH are growth hormone and the sex steroids. Schreck and Hopwood (1974) did not detect daily variations in androgen levels in the rainbow trout *Salmo gairdneri* but Leatherland

*et al.* (1973) demonstrated a daily rhythm in the growth hormone levels in *Oncorhynchus nerka*. Further investigations are necessary to clarify the mechanism of action of the GTH surges, and the patterns of temporal synergistic action of GTH and other hormones should be of concern to future workers. A correlation might exist between GTH surges, a possible daily rhythm in responsiveness of the gonads, and daily rhythms of hormones controlling either the availability of substances necessary for gonadal growth or the incorporation of these substances into the gonads.

Results from this study can be used to clarify the mode through which the goldfish uses environmental cues to regulate its reproductive cycle. The spawning period starts in May and can last about 45 days, depending on the environmental conditions (Yamazaki, 1965). The regressed fish used in this study in September are in the postspawning phase of their reproductive cycle and are possibly recovering from the physiologically exhausting process of breeding. Except for a slight temperature effect, they are apparently unresponsive to the different conditions of photoperiod and temperature imposed on them, in comparison to maturing and mature fish exposed to the same conditions. The patterns of serum GTH levels in a 24 hour period observed in these fish could represent the basal level of GTH secretion, unmodified by any stimulatory input to the hypothalamo-pituitary axis.

In the following months, the fish are undergoing ovarian recrudescence in preparation for the next breeding season (Yamazaki, 1965). The maturing fish in January had a GSI of about 6% and their ovaries had some yolk laden oocytes. These fish respond to different environmental regimes as is indicated by the fact that their patterns of serum GTH levels vary

depending on the environmental regime. Warm temperature and long photoperiod proved to be highly stimulatory for GTH secretion in maturing fish, as a large peak of serum GTH was detected 4 hours after the onset of light. It seems that under stimulatory conditions the fluctuations above the basal level of serum GTH are of a greater magnitude. Fish exposed to simulated winter conditions also showed fluctuations in serum GTH, but these fluctuations were smaller than those observed in the 16L:8D/21±1°C group. Therefore, the winter conditions allow the animals to slowly develop the gonads in time for the next spawning season.

In March, the fish are in the prespawning phase of their reproductive cycle (Yamazaki, 1965). The patterns of fluctuations in serum GTH levels again depends on the environmental regime. The stimulatory effect of long photoperiod and warm temperature on GTH secretion is less pronounced in mature fish than in maturing fish. Apparently, warm temperature alone has a particular effect, because regardless of photoperiod there was a peak in serum GTH levels 4 hours after the onset of light in both warm groups. The GTH surge on the day of ovulation described by Breton *et al.* (1972) in goldfish and Crim *et al.* (1975) in salmonids could represent the considerably increased peak of serum GTH described in the 16L:8D/21±1°C group of mature females. Such a large surge in GTH secretion would require the presence of conditions appropriate for spawning. The detailed pattern of GTH secretion during ovulation remains to be investigated.

## II. Pinealectomy-Blinding Experiment

The pinealectomy-blinding experiment was an attempt to gain some insight into the mechanisms by which light affects the fluctuations in

serum GTH levels in a 24 hour period. Photic information can reach the brain and possibly also the hypothalamus via the eyes, the pineal organ or possibly even other undescribed pathways. In this study, the effect of blinding, pinealectomy, or pinealectomy combined with blinding, were investigated. Only fish exposed to the 16L:8D/21±1°C regime were studied, with the samples taken at 1200 hr and 2000 hr because serum GTH levels are basal at 2000 hr and a peak in serum GTH occurs at 1200 hr in mature females under these conditions. Therefore, in this study the effect of pinealectomy and blinding on the occurrence of this peak in mature fish was investigated. The same experiment was also done on fishes with regressed gonads. Samples were taken at 1200 hr and 2000 hr so that the results from the regressed group and mature group could be compared. The 1200 hr and 2000 hr values from the pinealectomy-blinded experiment were also used as control references for the values of the 16L:8D/21±1°C group in the daily rhythm experiment. The fact that in all cases, the 1200 hr and 2000 hr values of the intact fish in the pinealectomy-blinded experiment, and the 1200 hr and 2000 hr values of the daily rhythm experiment, respectively, were not different, suggests that the results from the daily rhythm experiment are reproducible.

*Regressed fish*

In the regressed fish subjected to the 16L:8D/21±1°C regime, the pineal organ and the eyes do not seem to have any influence on the regulation of GTH secretion. Neither the removal of the eyes or the pineal organ significantly affected serum GTH levels at 1200 hr or 2000 hr, whether the GTH levels were expressed as absolute values or as changes from the presample. The fact that the GSI of the intact group

was not different from the GSIs of the other four experimental groups indicates that blinding and pinealectomy do not affect the development of gonads in the regressed fish. Fenwick (1970a) showed that pinealectomy did not have any effect on growth of gonads in goldfish in August and October, periods when the fish are insensitive to increasing daylength. This is in agreement with data reported here. Evidence presented in this study could also possibly indicate that the pineal organ is not involved in the mechanisms controlling the refractoriness of the hypothalamo-pituitary axis of regressed fish to short term exposure to stimulatory environmental conditions.

*Mature fish*

Data presented in this study suggest that the removal of the pineal organ causes abolition of the peak in serum GTH 4 hours after the onset of light in mature fish, but further investigations are required before this hypothesis can be firmly supported.

Although the 1200 hr values of serum GTH of the groups in which the pineal and/or the eyes were removed seem lower than the values of the intact and sham operated fish at 1200 hr, only the values of the group without the pineal (eyes are present) are significantly lower than the intact group. When GTH levels are expressed as changes from the presample, these trends are abolished. Some evidence in the literature supports the hypothesis proposed above. De Vlaming *et al.* (1977) determined diurnal variations in pituitary GTH activity in the cyprinid *Notemigonus crysoleucas*. The fish were collected in late March (mid-prespawning season) and the pituitaries were assayed after an exposure of 21 days to 15.5L:8.5D/15°C regime. The animals were therefore

in a similar stage of gonadal maturity and subjected to a similar environmental regime as the mature female goldfish used in this study. De Vlaming and co-workers showed that the GTH activity in the pituitary of sham operated fish is at a minimum at 1000 hr (4 hours after the onset of light) and at a maximum at 1600 hr. Assuming that the pattern of pituitary GTH values is inversely related to the pattern of serum GTH values, the minimum pituitary GTH value at 1000 hr could indicate a release of GTH at this time and possibly occurrence of a peak. On the other hand, the maximum pituitary GTH value at 1600 hr could indicate low levels of serum GTH. This pattern of secretion is similar to the pattern of serum GTH levels found in intact mature females in the present study. De Vlaming *et al.* (1977) also demonstrated that pinealectomy abolishes diurnal variations in GTH activity of the pituitary. This provides support for data presented in this study in which pinealectomy seems to abolish the occurrence of the peak in serum GTH 4 hours after the onset of light in mature females exposed to warm temperature and long photoperiod.

Some evidence is available in the literature suggesting that, in fish exposed to a long photoperiod, pinealectomy retards gonadal growth. De Vlaming (1975a) demonstrated that pinealectomy caused testicular involution or retarded ovarian development in the cyprinid *Notemigonus crysoleucas* maintained under a long photoperiod-warm temperature regime, in the prespawning season. The pineal organ seems to stimulate gonadal growth in the medaka, *Oryzias latipes* (Urasaki, 1973). The general hypothesis that the pineal organ stimulates gonadal growth in fish exposed to long photoperiod could be given a new interpretation using

the data presented in this study. Results from the daily rhythm experiment suggested that the daily surges of GTH might be physiologically more important than the basal GTH level and pinealectomy seems to abolish the peak in serum GTH in mature fish exposed to 16L:8D/21±1°C. This could signify that the inhibitory effect of pinealectomy upon gonadal growth in fishes maintained under long photoperiod could be the result of the abolition of daily fluctuations in serum GTH levels. Further investigations are required.

# SUMMARY

Goldfish having gonads in an immature state exhibit no fluctuations in serum GTH levels or fluctuations smaller in magnitude than those detected in female goldfish undergoing ovarian recrudescence or in females with a mature gonad.

The pattern of daily variations in serum GTH levels in females undergoing ovarian recrudescence and in mature females varies depending on the photoperiod and temperature regime, and tends to be similar when both these groups are subjected to the same set of environmental conditions.

Both temperature and photoperiod seem to be important factors involved in the regulation of the daily rhythm of GTH secretion.

The GTH levels detected in regressed goldfish may represent a basal level of GTH secretion, while the variations in GTH levels in the females undergoing ovarian recrudescence and mature females could represent fluctuations about this basal level. This is apparent since during at least a portion of the 24 hour period, the GTH levels of the three groups in different stages of sexual maturity and subjected to the same environmental conditions are similar.

Pinealectomy and blinding do not affect the daily variations in GTH levels in goldfish with a regressed gonad. Pinealectomy seems to abolish the daily variations in serum GTH levels in mature female goldfish, but further investigations are necessary before this can be established with certainty.

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